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Origins, Diversity, and Diversification of the Native Hawaiian Leafhoppers (Hemiptera: Cicadellidae: Nesophrosyne) and Their Obligate Endosymbionts

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Origins, Diversity, and Diversification of the Native Hawaiian Leafhoppers (Hemiptera: Cicadellidae: *Nesophrosyne*) and Their Obligate Endosymbionts

by

Gordon Morse Bennett IV

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Abstract

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Professor Patrick M. O'Grady, Chair

The relationship between plants and insects is one of the greatest evolutionary stories in the history of life on earth. Their importance in global terrestrial ecosystem functioning is self evident, as both represent the most abundant life on the planet. While plant-insect interactions have received much attention and are easily manipulated in experimental investigations, there have been few broad-scale phylogenetic studies for circumscribed herbivorous groups. As a result, the evolutionary role of interspecific interactions in promoting herbivorous insect diversification, at both the global and local scales, remains unclear. Remarkably, one of the largest gaps in our evolutionary and ecological understanding includes the sap-feeding insects in the Auchenorrhyncha suborder (Hemiptera), which contain some of the largest, terrestrially dominant host-plant restricted insect groups known (*e.g.,* Cicadas, planthoppers, and leafhoppers). The evolutionary success of Auchenorrhyncha is due, at least in part, to ancient associations with a consortium obligate bacterial endosymbionts that have persisted for over 260 million years. However, like their insect hosts, the diversity and evolutionary relationships of endosymbiont associations remain relatively unknown for most aucchenorrhynchan groups.

The leafhoppers (Cicadomorpha: Cicadellidae) remain one of the largest, yet poorly understood insect families. Of the 22,000 currently described species, thousands remain to be described with an overall unknown diversity (some estimates suggest as many as 90% of tropical Cicadomorpha remain to be described). This is surprising, since they offer excellent models to understand ecological and biogeographic mechanisms of species diversification due to their strict host-plant specificity, limited dispersal, and high rates of local endemism. The cicadellid subfamily, Deltocephalinae, represents the largest leafhopper groups, yet their patterns of species diversification, host-plant use, and endosymbiont associations remain almost entirely unknown.

This study used the Hawaiian Archipelago as a model system to investigate the roles of ecology, biogeography, and endosymbiont interactions in the diversification of the native Hawaiian leafhopper genus, *Nesophrosyne* (Cicadellidae: Deltocephalinae). The Hawaiian Islands offer a tractable natural laboratory to circumscribe and study plant-insect evolution due to their isolated, discrete and replicated nature, and high levels of endemism. *Nesophrosyne* represents one of the most diverse and ecologically dominant herbivore radiations on Hawai`i, but has eluded scientific attention for over 60 years. Species are obligate phloem feeders and are highly host-plant specific. Moreover, *Nesophrosyne* exhibits the quintessential characteristics of an adaptive radiation, including dramatic morphological adaptations to the endemic Hawaiian

flora and adaptive diversification across the archipelago to fill habitat types from coastal to sub-alpine regions. The specific goals of this study were to 1) update the current taxonomic status of *Nesophrosyne*, 2) determine the diversity and phylogenetic relationships of species in the genus, 3) infer the roles of ecology and geology in the adaptive radiation, historical biogeography, and species diversification dynamics of *Nesophrosyne*, and 4) to reconstruct the global relationships of the dual obligate bacterial endosymbionts of *Nesophrosyne* and their rates of evolution.

In the first chapter, the taxonomic history and status of *Nesophrosyne* was reviewed. The genus was redescribed, and the subgenus *Nesoreias* was synonymized with *Nesophrosyne*. Eight new species associated with the widespread host-plant species, *Broussaisia arguta* (Hydrangeaceae), were described. Results reveal morphologically cryptic diversity according to individual Hawaiian Islands and volcanoes within this group. A model usage of morphological and molecular characters was developed for future delimitation of species in *Nesophrosyne*.

The second chapter reconstructed a comprehensive phylogeny for *Nesophrosyne* in order to determine the origins, species diversity, and host-plant use of the native Hawaiian leafhoppers. Results support a monophyletic *Nesophrosyne*, originating from the Western Pacific basin, with a sister-group relationship to the genus *Orosius*. *Nesophrosyne* species are characterized by high levels of morphologically cryptic diversity and local endemicity, comprising > 200 species. Species demonstrate four dominant patterns of host-plant specialization that shape species diversity: 1) diversification through host switching; 2) specialization on widespread hosts with allopatric speciation; 3) repeated, independent shifts to the same hosts; and, 4) absence or low abundance on some hosts, suggesting herbivore interactions may limit ecological opportunity.

The third chapter inferred the roles of ecology and geology in the adaptive radiation, historical biogeography, and species diversification dynamics of *Nesophrosyne*. The molecular age of *Nesophrosyne* indicates a split from *Orosius* 4.5 million years ago (Ma), with a basal divergence on Hawai`i 3.2 Ma. The genus originated on Kaua`i and subsequently colonized younger islands as they formed. Ancestral host-plant reconstructions reveal that the plant families, Urticaceae and Rubiaceae, played important roles in the early diversification of *Nesophrosyne*. Results indicate that island geography have imposed significant barriers to continued gene flow, leading to extensive allopatric speciation and intra-island diversification. Finally, *Nesophrosyne* diversification dynamics show an initial burst in speciation rates, with a subsequent diversity-dependent decline, corresponding to island formation.

Finally, chapter four examined the global relationships of *Nesophrosyne's* dual obligate, bacteriome restricted bacterial endosymbionts, '*Candidatus* Sulcia muelleri' and a novel β-proteobacterium in the '*Ca.* Nasuia' genus. A global bacterial phylogeny was reconstructed, revealing a shared origin for the β-proteobacterial lineages throughout Deltocephalinae genera, and potentially throughout Auchenorrhyncha. The bacteriome association and transovarial transmission of *Nesophrosyne's* endosymbionts were confirmed using Fluorescent *in situ* Hybridization techniques. Finally, inference of absolute molecular rates demonstrates highly elevated rates of molecular evolution – the fastest so far recorded. We propose a second species in the genus *Nasuia* to describe the novel β-proteobacterium in *Nesophrosyne*.

Hawai`i has long been held as a model system to understand adaptive radiation and evolutionary biology, however my study is one of the first to test these patterns directly for a hyper-diverse endemic insect radiation, and for the suborder Auchenorrhyncha. The presented results illustrate that, in diverse herbivorous groups, multiple evolutionary processes play fundamental roles in species diversification, including associations with bacterial endosymbionts, host-plant specialization, insect-insect interactions, and the geologic formation

of islands. These results develop both an understanding of how ecological and geological controls shape adaptive diversification in insects, and a general model for contextualizing species diversification in herbivorous insects.

Dedication

I dedicate this to my folks, Gordon and Paula Bennett,
with love and admiration of their lives' accomplishments.

"Portions of that little journey bloomed with beauty. Occasionally we entered small basins walled in with low cliffs, carpeted with greenest grass, and studded with shrubs and small trees whose foliage shone with an emerald brilliance. One species, called the mamona (mamani), with its bright color, its delicate locust leaf, so free from decay or blemish of any kind, and its graceful shape, chained the eye with a sort of fascination. The rich verdant hue of these fairy parks was relieved and varied by the splendid carmine tassels of the Ohia tree. Nothing was lacking by the fairies themselves."

Mark Twain,

Mark Twain's letters from Hawai`i (1966, Univ. Haw. Press)

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CHAPTER 1

Review of The Native Hawaiian Leafhopper Genus *Nesophrosyne* (Hemiptera: Cicadellidae: Deltocephalinae) with Description of Eight New Species Associated with *Broussaisia arguta* (Hydrangeaceae).

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Abstract

A review of the native Hawaiian leafhopper genus *Nesophrosyne* (Hemiptera: Cicadellidae: Deltocephalinae) is presented. Specimens were examined from across the entire Hawaiian Archipelago, *Nesophrosyne* is redescribed, and the subgenus *Nesoreias* is synonymized with *Nesophrosyne*. Eight new species associated with the widespread host plant species *Broussaisia arguta* are described: *N. heopoko* sp.n. from Kaua'i; *N. makaihe* sp.n. from O'ahu; *N. magnaccai* sp.n. from Moloka'i; *N. broussaisiai* sp.n., *N. ogradyi* sp.n., and *N. kaupoi* sp.n. from Maui; and, *N. aakokohaikea* sp.n. and *N. kanawao* sp.n. from Hawai'i Island. Morphological and molecular characters were employed to delineate new species. Populations associated with *B. arguta* on different islands, individual volcanic mountains, and discrete geographic areas represent reciprocally monophyletic species. A monophyletic complex of five sibling species, morphologically cryptic on individual islands, were identified from Maui and Hawai'i Island. The *kanawao* species group is erected for these species and is further subdivided into two species subgroups based on monophyly, island endemicity, and morphology: *broussaisiai* species subgroup containing *N. broussaisiai, N. ogradyi,* and *N. kaupoi* on Maui; and, *aakokohaikea* species subgroup containing *N. aakokohaikea* and *N. kanawao* on Hawai'i Island.

Key Words: Hawaiian Islands, Sibling Species, Biogeography, Taxonomy, Endemism

Introduction

The native Hawaiian leafhopper genus *Nesophrosyne* Kirkaldy (1907) (Cicadellidae: Deltocephalinae: Opsiini) is a diverse and ubiquitous, yet understudied, element of the Hawaiian entomofauna. The native group currently comprises sixty-two described species (Kirkaldy 1907, 1910; Osborn 1935; Zimmerman 1948), distributed across the high islands of the archipelago (*e.g.*, Kaua'i – Hawai'i). The Hawaiian species are single island endemics, occurring in almost all habitat types from coastal scrub to sub-alpine regions (2500–3100 meters above sea level). Nearly all species are host plant specific, utilizing approximately 25% of the native Hawaiian plant genera, and 75% of the most species rich and ecologically dominant genera (*e.g.*, genera of the lobelloid group, *Myrsine*, *Hedyotis*, *Coprosma*: Kirkaldy 1907, 1910; Osborn 1935; Zimmerman 1948; Wagner *et al*. 1999).

Kirkaldy (1907) first described *Nesophrosyne* based on the presence of a large median anteapical cell and a small outer anteapical cell on the forewing. He later expanded the description to include more detail on forewing cell number and size, venation, and the relative size of the pronotum, which resulted in splitting the genus into two subgenera, *Nesophrosyne* and *Nesoreias* (Kirkaldy 1910). The latter subgenus being separated by the complete absence of the outer anteapical cell (Fig. 1). The shape of the outer anteapical cell is extremely plastic in size

and presence, negating the subgeneric taxonomic classification. Unfortunately, Kirkaldy's treatments further complicated taxonomy within *Nesophrosyne* by issuing cursory descriptions of forty-one species. These descriptions, sometimes limited to only a few sentences, highlighted color and other variable traits and are inconsistently based on either males or females, which can show dramatic sexual dimorphism. Kirkaldy (1910) also neglected to provide illustrations and a key to these species.

Osborn (1935) described twenty-one new species, and redescribed thirty-seven of the species originally proposed by Kirkaldy (1910). The redescriptions provide clarity to the taxonomic issues presented by the shortcomings of Kirkaldy's (1907, 1910) treatments. While Osborn's species descriptions are more comprehensive with excellent illustrations and discussion of phylogenetic relationships, their utility is somewhat limited. Osborn's (1935) descriptions were made from either females or males, focused on highly variable characters, failed to include internal genitalia, and did not provide a key to separate species (except for *Nesoreias*). Osborn's circuitous discussion on crown evolution and similarities in wing venation to "*Scaphoideus*-like" genera did not provide any further insight into morphological characters uniting species in *Nesophrosyne*.

Zimmerman (1948) synthesized previous work on *Nesophrosyne*, including for the first time, a short discussion of a bifurcate aedeagus with slender hooked apical processes and drawings of internal genitalic characters. Unfortunately, Zimmerman did not extend full descriptions of external or internal genitalia beyond the type species, offering only a single synonymy. He did provide an ambitious key to the *Nesophrosyne* species based largely on coloration and relative sizes, which is difficult to use in some cases. Cognizant of the challenges in treating *Nesophrosyne*, Zimmerman (1948) paused to presciently note of his key (and taxonomic state of the genus) that "*Nesophrosyne* [presents a] confusing natural complex of complexes… I realize [the key is] difficult to use, and it may not work for all species or all specimens of a single species." Despite the difficulties, Zimmerman's (1948) treatment it provides a good starting point for identifying Hawaiian *Nesophrosyne* and its species diversity.

Ghauri (1966), in a revision of the genus *Orosius* Distant, provided a more thorough genus level redescription of *Nesophrosyne*. He included a description of the internal genitalia in an attempt to resolve phylogenetic relationships between the genera and to establish their taxonomic status (see also Linnavuori 1960a, 1960b, 1975). However, Ghauri's (1966) redescription was restricted to the type species of the genus, and his treatment falls short of describing the morphological diversity found among *Nesophrosyne* species. For example, some characters used to delineate *Nesophrosyne* (*e.g.*, apical processes of the aedeagal arms) are variable or absent in some species, others are seemingly erroneous (*e.g.*, posterior processes of styles not hooked, and the convergence of gonoducts at base of aedeagus; see Figs. 4–6 & 8–9), and utilize antiquated terminology.

Other species outside of the Hawaiian Archipelago have been placed in the genus *Nesophrosyne* at various points. *Orosius ryukyuensis* Ishihara (1965) and *Orosius argentatus distans* Linnavuori (1960b) were described as members of *Nesophrosyne*. Linnavuori (1975) and Ghauri (1966) moved both species to *Orosius*, respectively. *Orosius filigranus* (Dlabola 1964) and *O. cellulosa* Lindberg (1958) were moved from *Thamnotettix* to *Nesophrosyne,* and were subsequently transferred to *Orosius* by Ghauri (1966). Linnavuori (1960a, 1960b) treated *Nesophrosyne*, synonymizing it with *Orosius* (even considering *Orosius* a subgenus of *Nesophrosyne*). However, Ghauri (1966) split the two genera back into their previous designations, which was later confirmed by Linnavouri (1975), removing all of the non-

Hawaiian taxa from *Nesophrosyne*. Thus, the genus *Nesophrosyne* currently refers to the lineage endemic to the Hawaiian Archipelago.

Aside from Ghauri's (1966) generic level redescription, *Nesophrosyne* has remained untreated for over 60 years. Much remains to be understood, and previous work has left some taxonomic confusion that must be overcome in order to provide reliable species concepts and identification keys to species. *Nesophrosyne* needs to be more thoroughly circumscribed, with a complete description of the morphological variation present in the group with updated terminology. Species descriptions need to be made consistent. The mixed gender of Osborn's (1935) and Kirkaldy's (1910) descriptions and type specimens make positive identification of some species difficult. Described species need to be redescribed to include internal genitalia, which provide a more reliable suite of morphological characters for species determination. Moreover, *Nesophrosyne* is far more diverse than previous work suggests, with many species waiting description (Fig. 2: See Kaua'i, Maui, and Hawai'i). Once species have been more thoroughly treated, comprehensive, user-friendly keys need to be devised.

Here, we provide a review of *Nesophrosyne* based on specimens collected from across the Hawaiian Islands. A redescription of the genus with updated terminology is included to encompass morphological variation in external and internal genitalic characters. We examined wing venation and molecular characters within *Nesophrosyne* and conclude that *N.* (*Nesoreias*) is a synonym of *N.* (*Nesophrosyne*). Finally, we use morphology, geography, and molecular tools to delineate and describe eight new species associated with the host plant *Broussaisia arguta*.

Materials and Methods

Taxonomy

Collection permits were obtained from the State of Hawai'i Department of Land and Natural resources, Division of Forestry and Wildlife for access to state lands and Natural Area Reserves; and from the National Park Service for access to Hawai'i Volcanoes National Park and Haleakala National Park. Permits were also obtained from the following private landowners: East Maui Irrigation, The Maui Land and Pineapple Company, and Parker Ranch.

Specimens were collected by sweep netting and placed directly into 95% ethanol. Specimens were field-sorted based on geographic collection locality and plant associations, and given a four-digit decimal collection barcode (*e.g.,* GB-006.7) for reference. Material was returned to U.C. Berkeley for identification, description, and molecular analyses (see below). Specimens were examined, photographed, and measured using Nikon SMZ1500 microscope with a DS-L1 imaging system. Wing venation was examined for all specimens collected from across the islands in an effort to assign species to a subgenus. Entire abdomens were removed, and a small cross section was saved in 95% ethanol for DNA extraction. Remaining abdominal segments were incubated overnight in 10% potassium hydroxide solution at room temperature. Cleared genitalia were placed in glycerin in plastic genitalia vials and pinned with specimens.

Species identities for fifty-two of the previously described species were determined. Thirty-two type specimens deposited in the Bernice P. Bishop Museum (BPBM), Honolulu, HI, were examined. The remaining types are in the British Museum. Twenty species, determined by Osborn, Zimmerman, Perkins, and other workers, were examined from the BPBM and U.H. Manoa Entomology Museum (UHM). The non-type material examined from the BPBM and

UHM collections covers a majority of the type specimens deposited in the British Museum. In addition ~1500 specimens, including twenty-one described species and many undetermined species from D.A. Polhemus' personal collection were examined. The Polhemus material is on loan from the Smithsonian Institute, Washington D.C. The types of three species, *N. ignigena, N. nimbigena and N. insularis*, are missing (Osborn 1936; Zimmerman 1948) and were not examined, however specimens determined as these species by Perkins exist in the Bishop Museum and have been examined.

The *Nesophrosyne* type material has been treated twice (Osborn 1935; Zimmerman 1948), and the resultant published resources are adequate to determine specimens of newly collected material and to designate new species. Osborn's (1935) descriptions, redescriptions of Kirkaldy's types, and included drawings adequately treat the external morphology of all *Nesophrosyne* species, and are usable for species determinations. He failed to treat two type specimens in the British Museum, but Zimmerman (1948) reviewed them. Zimmerman (1948) later treated the Hawaiian *Nesophrosyne* and many of the type specimens, as well as synthesizing Kirklady's (1907, 1910) and Osborn's (1935) treatments. He accounted for each of the type specimens and their locations, and effectively redescribed them all by generating a workable master key and island-by-island keys to each of the species. Zimmerman (1948) also included photographs and drawings of nearly all of the described species and their types.

Collected specimens were identified to species using Zimmerman's (1948) key to the described species, and Osborn's (1935) comprehensive species treatments, with included redescriptions of nearly all of Kirkaldy's described species (1907, 1910). They were further compared against redescriptions and a key to the thirty-two species in the type collection at the BPBM (Bennett unpub). Species unable to be identified with these resources and representative of new ecological and host plant associations are considered to be new. Terminology for new species descriptions follows Dietrich (2005) based on Oman (1949), and Dietrich and Dmitriev (2007). Holotypes and a series of paratypes have been deposited in the BPBM, Honolulu, HI.

Molecular Analyses

The molecular data and phylogenetic analyses presented here are preliminary results of a larger systematics research project, which will be published elsewhere. Molecular analyses are used to strictly reinforce morphological results and address the phylogenetic placement of species in the subgenus *Nesophrosyne* (*Nesoreias*), the polyphyly of species associated on *Broussaisia arguta*, and the cryptic nature and monophyly of the *kanawao* species group. Methodological details pertinent to the presented results are outlined below. Specific laboratory procedures and resultant data (*e.g.,* DNA extraction, PCR amplification, individual sequence data, etc.) will be published elsewhere.

Taxonomic sampling for molecular analyses include 107 *Nesophrosyne* terminals representing 75 endemic Hawaiian species, and three outgroups, *Nesophyla variata* Osborn*, Nesophyla sp. and Deltocephalus sp.,* from the Marquesas Islands, French Polynesia. The island sampling distribution of Hawaiian *Nesophrosyne* is as follows: Kaua'i = 11 spp.; O'ahu = 19 spp.; Lana'i = 1 spp.; Moloka'i = 3 spp.; Maui = 24 spp.; Hawai'i = 17 spp.

Four genes, two mitochondrial (mtDNA: Cytochrome Oxidase II (COII) & 16S) and two nuclear (nucDNA: Histone 3 & Wingless), were used to reconstruct phylogenetic relationships in *Nesophrosyne*. Primer sequences were obtained from previous studies: COII, Takiya *et al*. 2006 ;16s, Dietrich *et al*. 1997; Histone 3, Ogden & Whiting 2003; and, Wingless, Cryan *et al*. 2004.

The three protein-coding genes (COII, Histone 3, and Wingless) sequences were conceptually translated in MacClade (Maddison & Maddison 2003) and manually aligned against an annotated GenBank sequence. The 16S ribosomal dataset includes stems and loop regions that are non-trivial to align. To deal with this, we used Probalign v1.2. accessible through the CIPRES web portal (Miller *et al*. 2009), which optimizes pairwise posterior probabilities of sequence residues with a partition function approach (Roshan & Livesay 2005). Probalign was chosen based on its superior performance over other available alignment programs. Base pair positions for which statements of homology are questionable were removed from downstream analyses. The total concatenated data set was 1914 base pairs in length.

Uncorrected pair-wise distances for COII were estimated in PAUP* (Swofford 2002). COII was used as an alternative to the commonly used Cytochrome Oxidase I (COI) in assessing molecular divergence as further evidence for species hypotheses (Savolainen *et al.* 2005). Repeated attempts to sequence COI with previously published primer sequences (Simon *et al*. 1994) and multiple primer pairs designed for this study failed. Phylogenetic trees were constructed using Maximum Likelihood. Models of evolution were estimated in Modeltest 3.7 using the Akaike Information Criterion (Posada & Crandall 1998). Phylogenetic analyses were performed in RAxML-VI-HPC v7.2.5 on the Abe server accessible through Cipres Portal (Stamatakis 2006, 2008; Miller *et al*. 2009). Runs were performed with 100 bootstrap partitions under the GTR+I+Γ model of nucleotide substitution for two data partitions: mtDNA and nucDNA. Runs were restarted three times to confirm that searches converged on the same posterior optima.

Results and Discussion

Nesophrosyne and Nesoreias

We examined wing venation in a total of 1085 specimens, in at least 107 distinct morphological species from across the Hawaiian Archipelago (See Appendix 1 for annotated list of species examined). The examined specimens confirm that wing venation is highly variable not only within the genus, but also within species and even single individuals. Presence of the outer anteapical cell is the most common state, however variation in the size, shape, and presence (in one or both wings) is common in large populations. In some rare instances, the cell is subdivided with individuals having up to four outer anteapical cells. The character's extreme variability demonstrates the lacking predictive, evolutionary functionality necessary to define a cohesive subgenera, or taxonomic grouping. Thus, the outer anteapical cell cannot be used to differentiate subgenera of *Nesophrosyne*.

Molecular phylogenetic analyses reveal *Nesoreias* as polyphyletic, falling out in at least three clades (data not shown). *Nesophrosyne eburneola* Osborn 1935 and *N. marginalis* Osborn 1935 are placed as sister species with high support, however *N. insularis* Kirkaldy 1910 and *N. sp.1* are nested well within two different clades with high support. The distribution of species with individuals without cells, with one cell, or with extreme size variation is random across the tree with high support for some groups. These molecular data, coupled with genus-wide morphological assessment, indicate that the subgenus *Nesoreias* is dubious and we synonymize it with *Nesophrosyne.*

Delimitation of New Species Associated with Broussaisia arguta

Broussaisia arguta (Gaud. 1830) (Hydrangeaceae) belongs to a monotypic genus endemic to the Hawaiian Islands. It grows as a shrub predominately in wet forest habitats, ranging in elevation from 370m–2070m (Wagner *et al.* 1999). *Nesophrosyne* species are commonly found on *B. arguta* across the archipelago. Except for Hawai'i Island, no species have been documented or described exclusively from *B. arguta*. Zimmerman (1948) indicated a single collection of *Nesophrosyne pluvialis* Kirkaldy 1910 on *B. arguta* and two other hosts (*Coprosma sp*. and *Acacia koa*). *N. pluvialis* was redescribed as occurring on the genus *Coprosma* (Osborn 1935), and recent collections have found it only on *Coprosma sp.* from multiple collection localities and over several years (Bennett unpub). The collection record referred to by Zimmerman (1948) is likely a chance collection.

Nesophrosyne species collected from *B. arguta* do not match any described by Kirkaldy (1907, 1910) or Osborn (1935), are unable to be keyed (Zimmerman 1948; Bennett unpub), and are distinct from the type specimens redescribed by Osborn (1935) at the British Museum and Bennett (unpub) at the Bishop Museum. Examination of morphology combined with molecular results show eight unique species occurring on Kaua'i (1 sp.), O'ahu (1 sp.), Moloka'i (1 sp.), Maui (3 sp.), and Hawai'i (2 sp.). Species on Kaua'i, O'ahu, and Moloka'i are morphologically distinct from each other and from species on Maui and Hawai'i Island, indicating independent switches to *Broussaisia arguta*. Species on Maui and Hawai'i Island show strong similarities for internal male genitalia (*e.g.,* the unique structure of the connective), supporting monophyly of this group. Populations occurring on different volcanic mountains on a single island (*e.g.,* Hawai'i Island: Kohala Range and Mauna Loa) are difficult to distinguish and morphologically cryptic, but do show subtle variation in external morphology, and in some cases in internal male genitalia (Maui: West Maui and East Maui Haleakalā leeward face). The species assemblages on Maui and Hawai'i are easily discernable from one another by external morphology and pigmentation (*e.g.,* Hawai'i Island species being darker). The species of the *broussaisia* subgroup (Maui) can be separated by a combination of claval pigmentation and the reduced aedeagal processes in *N. kaupoi*. *Nesophrosyne broussaisiai* (West Maui) represents a darker form than *N. ogradyi* and *N. kaupoi* (East Maui). While there is some overlap in the species of the *aakokohaikea* species subgroup (Hawai'i Island), they can usually be separated by external claval pigmentation, as *N. kanawao* is generally paler than *N. aakokohaikea*.

Partitioned likelihood phylogenetic analyses (Fig. 3), combined with COII uncorrected pairwise distances (Table 1), provide strong support for species hypotheses. Results demonstrate that there are unique species on each island, with evidence for at least three switches to *B. arguta* as a host plant (see species descriptions for more detail). Species from Hawai'i Island and Maui represent a monophyletic lineage, with highly supported geographic sub-structuring (Fig. 3). Results confirm that geographically separated species co-occurring on single islands, although morphologically cryptic, are reciprocally monophyletic sister lineages. Uncorrected pairwise distances for COII (Table 1) demonstrate relatively high sequence divergence between these lineages: 4.2% – 11.24% (percent divergence within populations = 0.0% – 1.82%). These genetic breaks are separated according to volcano formation and unique geographic provenance (*e.g.,* Haleakalā leeward face, Maui), which conform to potential discontinuities in *B. arguta*, ecological, and climatological ranges.

Review of molecular evidence, morphology, and geography indicate that there are at least eight new species associated with *B. arguta*, described below. Molecularly distinct populations

on Maui and Hawai'i Island, represent morphologically cryptic sibling species deserving of individual species demarcation (Bickford *et al.* 2006; de Queiroz 2007). We recognize sibling species for their important information about the biotic diversity and endemicity of the Hawaiian entomo-fauna, and to avoid future taxonomic confusion presented by cryptic species (Bickford *et al.* 2006).

Taxonomy

Nesophrosyne Kirkaldy 1907: 160

Type Species: *Euttetix perkinsi* Kirkaldy 1907: 160
Nesophrosyne (*Nesoreias*) Kirkaldy 1910: 573 **new synonymy**

Diagnosis:

Moderately robust leafhoppers. Ocelli visible in dorsal view. Forewing with central and small outer anteapical cell, outer cell variable. Clypeus broad, with margins straight. Pygofer with 16 or fewer macrosete and fine stout microsete on posterior half of apical lobe. Subgenital plate elongate, curved posterodorsad; lateral edges hirsute. Aedeagus bifurcate with two aedeagal arms and gonopores, curved posterodorsad; aedeagal arms with apical processes; base of gonoducts forming two preatria. Styles with well-developed and hooked posterior processes, curved posterolaterad; base of posterior processes with medial ledge with fine microsete. Connective 'Y' shaped. Endemic to the Hawaiian Archipelago.

Description:

Dorsum (see Figs. 4–9): Small slight (3.25 mm) to large robust (5.5 mm) leafhoppers. Color and coloration patterns varying widely. Crown ranging from bluntly rounded to elongate and extremely produced. Ocelli visible in dorsal view, situated on margin of crown and face. Pronotum as wide as, or in some cases slightly wider than, head; anterior margin rounded, convex; posterior margin straight. Forewing with large central anteapical cell and smaller outer anteapical cell; central anteapical cell narrowed medially; outer anteapical cell variable in size and shape from nearly the length of the central anteapical cell to completely absent (varies within and between species). Clavus of many species with well-formed, conspicuous saddle mark (Fig. 7).
Venter: Clypeus broad, width nearly 3/4 length; lateral margins not sinuate. Clypellus elongate, lateral margins subparallel.
Genitalia: Pygofer with 16 or fewer macrosete on posterior half of apical lobe, extending beyond posterior edge; fine stout microsete covering posterior half of apical lobes (Figs. 4–6b & 8–9b). Valve rounded, notched symmetrically on either side of posterior apex. Subgenital plate elongate, curved posterodorsad, tapering towards distal point; lateral edges hirsute from base to apex, with long filamentous and macrosete (usually 5) interspersed (not unique to this genus). Anal tube long, not fully membranous dorsally, with well sclerotized band; laterally and ventrally sclerotized. Aedeagus bifurcate with two aedeagal arms and gonopores; aedeagal arms curving posterodorsally, height of arms variable from sub-anal tube to terminating at the dorsal edge of pygofer; gonopore near apex, usually subapical, opening posteromesially; aedeagal arms

with produced apical processes hooked anterolaterad, length from short-straight (~1/10 length of aedeagal arm) to long and recurving (~1/4–1/5 length of aedeagal arms), completely absent in some species; base of gonoducts not connected, forming two preatria; anterior base of aedeagus with apodeme pointed dorsad (Figs. 4–6c,f,g & 8–9c,f,g). Styles with well-developed posterior processes, curved posterolaterad; preapical lobe with fine microsete (Figs. 4–6e & 8–9e). Connective 'Y' shaped; body thinning medially, appearing hourglass shaped; anterior arms variably shaped and splayed (Figs. 4–6d & 8–9d).

Material Examined:

A total of 1085 specimens from at least 101 distinct morphological species were examined. Sampling from individual islands is as follows: Hawai'i Island, males = 232, females = 301, species = 32; Maui, males = 118, females = 93, species = 32; Lana'i, males = 1, females = 3, species = 3; Moloka'i, males = 18, females = 12, species = 4; O'ahu, males = 59, females = 37, species = 15; Kaua'i, males = 105, females = 106, species = 16.

Discussion:

Despite intraspecifc variation in *Nesophrosyne*, color patterning offers useful characters for species identification (we refer readers to Zimmerman's (1948) well-illustrated review of *Nesophrosyne* for a photographic menagerie of the genus' external morphological diversity). However, external and internal genitalia provide a more reliable suite of morphological characters for species determination. These characters, combined with molecular data and species' geographic ranges, provide congruent lines of evidence for *Nesophrosyne* species and cryptic species groups. Results presented here provide evidence of cryptic sibling species, which may be common in other lineages associated with widespread host plants. Molecular characters may be necessary for identification of species within these groups.

Nesophrosyne's biogeographic origin and relationships to other Pacific cicadellid genera are currently unknown. Kirkaldy (1907, 1910) proposed a relationship to *Nephotettix*, but he did not explain the basis for this hypothesis. Osborn (1935) strongly argued for a *Scaphoideus*-like ancestor "derived from either America or Asia…, [colonizing] during the early history of life on the islands."

A plausible origin for the Hawaiian *Nesophrosyne* lineage is the genus *Orosius* found along the western Pacific Rim including Japan, Southeast Asia, and the Oceania region. Linnavuori (1960a, 1960b, 1975) first examined this relationship, and based on similarities in the bifurcate aedeagus, blunt crown and flecked forewing of some *Nesophrosyne* species, presence of an elongate outer anteapical cell, hollowing near the base of the antennae, and overlapping shape and size of the frontoclypeus of some species, he combined the genera with *Orosius* as a subgenus of *Nesophrosyne*. Ghauri (1966) later reexamined Linnavuori's conclusions, determining that morphological differences, including the anal tube structure, subgenital plate shape, divergence of frontoclypeus shape in some species, and hooking distal process found only in *Nesophrosyne* were sufficient to preserve *Nesophrosyne* and *Orosius* as separate genera. Linnavuori (1975) conceded, commenting that a thorough investigation of the biotic and morphological diversity of both genera is necessary to resolve the question. He further reiterated the opinion that *Nesophrosyne* originated from eastern Oceania. Both Ghauri (1965) and Linnavuori (1975) noted the presence of hooking apical processes on the aedeagal arms in

Nesophrosyne, which are absent in *Orosius*. A more thorough investigation of *Nesophrosyne* genitalia reveals that the apical processes are absent in some species and the genitalic affinities between the two genera are stronger than previously thought. The relationship needs to be examined phylogenetically.

Key to *Nesophrosyne* species associated with *Broussaisia arguta* on the Hawaiian Archipelago

Note: In most cases knowledge of host plant and collection locality (island and volcanic mountain) alone will differentiate species associated with *Broussaisia arguta*.

1. Head obliquely triangulate (Fig. 4a), with crown produced medially, bluntly pointed. Kaua'i. ……..……...…….. *N. heopoko sp. nov.*

- Head not triangulate, crown weakly produced (Figs. 5–6a & 8–9a), rounded apically. ……..……..……..... 2

2. Gonopores subapical; aedeagus with apical processes produced distally, elongate 1/4–1/5 length of aedeagal arms (Figs. 4 & 5f). ……...……..…… 3

- Gonopores apical; apical processes of aedeagus extending from pore, short (~1/10 length of arm) or absent (Figs. 8 & 9f); Maui and Hawai'i. ……...…….…… *kanawao* species group, 4

3. Dorsum with well-formed saddle mark, tapering to point anterad; posterior end subflat, appearing arrow-like pointing anterad; O'ahu (Fig. 5). ...……...…… *N. makaihe sp. nov.*

- Species without any pale markings on clavus; forewing veins prominent, darkly pigmented; Moloka'i (Fig. 6). …….....…….....… *N. magnaccai sp. nov.*

4. Dorsum with central pale saddle mark always present, usually with paired pale blotches anterad, appearing as three pale marks showing bilateral symmetry; palest forms with clavus entirely pale; Maui (Fig. 7 & 8). …….....… *broussaisiai* species subgroup, 5

- Dorsum darker than above, usually without conspicuous saddle mark; claval veins discontinuously pale, extending short length from commissural line (appearing as two pale lines curving anterad) and with anterior intersection of claval veins pale, showing bilateral symmetry; palest forms with irregular, large pale blotches throughout clavus; Hawai'i Island (Fig. 7 & 9). ……..……..... *aakokohaikea* species subgroup, 7

Note: The following are a closely related assemblage of *Nesophrosyne* species most easily discerned by knowledge of collection locality:

5. West Maui; almost always with three pale marks (Fig. 7a); darkest forms with only central oval saddle mark; apical process present, short 1/10 length of aedeagal arm. ……..……...……..... *N. broussaisiai sp. nov.*

- East Maui; characters not as above. ……...……...…… 6

6. Claval coloration ranging from three mark forms to entirely pale; apical process present, short 1/10 length of aedeagal arm (Fig. 7 & 8).
……..…….....…. *N. ogradyi sp. nov.*
- East Maui, Haleakalā leeward face; species with claval coloration usually entirely pale; darker forms with dark bands across clavus, appearing disrupted; apical processes absent.
…….……..... *N. kaupoi sp. nov.*

7. Claval veins partially pale, extending from commissural line (appearing as two pale line curving anterad), with anterior intersection of claval veins pale, showing bilateral symmetry (Fig. 7 & 9). …...…….……..……...…… *N. aakokohaikea sp. nov.*

- Clavus predominately pale, appearing irregularly shaped, connected with thin pale line across commissural line. ……...…… *N. kanawao sp. nov.*

Nesophrosyne heopoko Bennett sp. nov.

(Fig. 4a–g)

Diagnosis:

Length: male = 3.94mm, female = 5.03mm. Brown-copper colored species. Head obliquely triangulate with crown produced; posterior end conspicuously concave. Forewing venation dark except on clavus. Clavus without saddle mark, veins partially pale extending from commissural line. Pygofer rounded; posterior-dorsal edge convex. Aedeagus with preapical gonopore; apical processes short and straight. Style with depressed preapical lobe. Endemic to Kaua'i.

Description:

***Dorsum*:** Brownish-copper colored species (Fig. 4a). Head triangular with oblique vertices; crown produced and rounded with articulated dark markings, separating the anterior 1/3 into a pale triangular shape, two lateral dark dots at 2/3 distance from crown divided by coronal suture. Pronotum predominately pale; anterior margin infused with dark blotches; darkest forms showing faint 'V' form, extending to posterior margin. Mesonotum predominately pale; mesonotal triangles darkly colored and edged with orange. Scutellum pale. Forewing cells hyaline; veins darkly colored throughout; darkest forms with cells infused with dark pigmentation; outer anteapical cell present, triangular. Clavus without prominent saddle mark; veins partially pale extending from commissural line, curved anterad.
Venter: Face with clypeus pale, with dark coloration restricted to posterior lateral margins; clypellus varying between posterior half dark to completely dark; lorum edged in dark, with darkest forms being dark throughout; gena pale, with antennal ledges and margin along eye dark. Abdominal segments predominately dark, with thin pale line along each posterior margin; pleurites partially dark on anterior half. Legs mostly pale, with femora and tibiae partially dark; bases of macrosetae dark.
Genitalia: Pygofer (Fig. 4b) bluntly pointed at mid-length, appearing rounded; ventral lobe reduced and rounded, edge to apex slightly rounded at base then straightening; posterior-dorsal edge convexly rising from apex; dorsal edge flat; 7 macrosete. Aedeagus (Fig. 4c, f, g) with bifurcate aedeagal arms relatively close in width, rising conspicuously higher than central

apodeme, arms slender; gonopore preapical; apical processes distad of gonopore, tapering and hooking at apex, short and straight (difficult to see without removing internal genitalia). Style (Fig. 4e) posterior processes curved, widening at base; preapical lobe, with microsete extending from depression and along dorsal groove. Connective (Fig. 4d) elongate, relatively close in length to styles; anterior edge slightly depressed; width between posterior arms narrowed.

Distribution: USA: Hawaiian Islands, Kaua'i, North West Kōke'e State Park, >1220m (Fig. 3).

Measurements:

Body length: Male (n=6) = 3.94mm (3.80mm–4.10mm); Female (n=3) = 5.03mm (4.90mm–5.20mm). ***Genitalia*** (n=5): Pygofer = 0.48mm (0.47mm–0.50mm); Style = 0.37mm (0.36mm–0.39mm); Connective = 0.32mm (0.31mm–0.33mm); Aedeagus lateral length = 0.22mm (0.21mm–0.25mm); Aedeagus posterior height = 0.26mm (0.25mm–0.27mm).

Material examined:

Type material: Holotype: 1 male, Hawaiian Islands, Kaua'i, Kōke'e Rd (State Highway 550), N22^{o} 07.125, W159^{o} 38.016, Elevation: 1270m, 17May2007. Host Plant: *Broussaisia arguta*, Coll. G.M. Bennett and K.M. Magnacca. Deposited in the BPBM, Honolulu, Hawai'i. Type #: 17301. ***Additional material***: 5 males, 3 females, same as holotype. Deposited in the BPBM, Honolulu, Hawai'i

Etymology:

Heopoko is Hawaiian for short-tipped end of penis: *Poko* is the adjective for short, and *Heo* is the noun for the tip of the penis. The name was chosen to describe the short apical processes of the aedeagal arms.

Discussion:

One species is described from Kaua'i occurring on *Broussaisia arguta*. Extensive sampling through topographically difficult areas would be required to test if there are other species occurring on this island. However, Kaua'i represents a relatively low, single volcanic mountain reducing the probability of other lineages.

Phylogenetic results indicate that *N. heopoko* is unrelated to other species associated with *B. arguta*, representing an independent shift to the host plant. *Nesophrosyne heopoko* is morphologically similar and closely related to *N. sp.1* (Fig. 3; COII % divergence avg. = 15.01%), co-occurring in the same range on the host plant *Pipturus sp.*. They are easily distinguished from each other using head shape, length of aedeagal distal processes, and absence of the outer anteapical cell in *N.sp.1*.

Nesophrosyne makaihe Bennett sp. nov.

(Fig. 5a–g)

Diagnosis:

Length: male = 3.74mm, female = 4.97mm. A dark species, with a prominent saddle mark resembling a spear point, pointed anterad. Crown produced and bluntly rounded. Pygofer with a pointed apex at 2/3 height from base. Aedeagus with long apical processes, recurving anterolaterally 1/4 length of aedeagal arms. Endemic to O'ahu, Wai'anae Mountain Range.

Description:

Dorsum: Dark species with prominent pale-yellow markings, extending from clavus through mesonotum (Fig. 5a). Crown and pronotum dark; lighter forms with pale light brown blotches between ocelli. Mesonotum mesially pale; mesonotal triangles dark, extending to lateral margins. Scutellum pale. Forewing predominantly dark with cells and veins obfuscate in dark pigmentation; costal cells along posterior half clear, divided by a darkly pigmented R1 vein; outer anteapical cells present, triangular. Clavus with large conspicuous saddle mark, widest at base and tapering anteriorally, resembling a spear point.
Venter: Face with well-formed grill pattern on clypeus, divided by central dark latitudinal line, posterior half entirely dark; clypellus, lorum, and gena dark. Abdominal segments predominately dark with thin pale line along each posterior margin; pleurites partially dark on anterior half. Legs almost entirely pale; hind femora dark anteroventrad; hind tarsal segments dark at joints.
Genitalia: Pygofer (Fig. 5b) produced and pointed, rising at 2/3 height from base; ventral lobe produced with long flat edge, angled posteroventrad, giving rise sharply to apex; posterior-dorsal edge rising form apex rounded; posterior edge flat; 11 macrosete. Aedeagus (Fig. 5c,f,g) with aedeagal arms widely splayed, rising above central apodeme; gonopore preapical; apical processes long approximately 1/4 length of aedeagus, distad of gonopore, tapering before hooking anterolaterally. Style (Fig. 5e) large; posterior processes short; preapical lobe flat and angular with microsete. Connective (Fig. 5d) short; posterior edge notched; anterior appendages splayed widely.

Distribution: USA: Hawaiian Islands, O'ahu, Western Wai'anae Mountain Range, ~1220m, wet forest.

Measurements:

Body length (n=8): Male (n=8) = 3.74mm (3.60mm–3.90mm); Female (n=10) = 4.97mm (4.80mm–5.10mm). ***Genitalia*** (n=6): Pygofer = 0.48mm (0.47mm–0.50mm); Style = 0.38mm (0.37mm–0.39mm); Connective = 0.28mm (0.27mm–0.29mm); Aedeagus lateral length = 0.26mm (0.24mm–0.27mm); Aedeagus posterior height = 0.25mm (0.23mm–0.27mm).

Material examined:

Type material: Holotype: 1 male, Hawaiian Islands, O'ahu, Wai'anae Mountains, Mt. Ka'ala, Summit Bog Boardwalk, N21^{o} 30.504, W158^{o} 08.865, Elevation: 1210m, 29Aug2009. Host

Plant: *Broussaisia arguta.* Coll. G.M. Bennett, K. Magnacca, and D.A. Polhemus. Deposited in the BPBM, Honolulu, Hawai'i. Type #: 17302. ***Additional material***: 22 males, 12 females, Hawaiian Islands, O'ahu, Wai'anae Mountains, Mt. Ka'ala, Summit Bog Boardwalk, N21^{o} 30.504, W158^{o} 08.865, Elevation: 1210m, 26May2007, 27July2009, and 29Aug2009. Host Plant: *Broussaisia arguta*. Coll. G.M. Bennett, K. Magnacca, and D.A. Polhemus. Deposited in the BPBM, Honolulu, Hawai'i. 21 males, 7 females, Hawaiian Islands, O'ahu, Wai'anae Mountains, Mt. Ka'ala, Summit Bog Boardwalk, N21^{o} 40'40" W158^{o} 08'48", Elevation: 1220m, 6May2000. Host Plant: *Broussaisia arguta*. Coll. D.A. Polhemus. Deposited in D.A. Polhemus's personal collection at the Smithsonian, Washington D.C.

Etymology:

Makaihe is a Hawaiian phrase for spear tip: *Maka* is the noun for point or tip of blade, and *Ihe* is the noun for spear. The name was chosen to describe the resemblance of the claval saddle mark to that of a spear point.

Discussion:

A single species is described for the *Nesophrosyne* occurring on *Broussaisia arguta* on O'ahu. Three specimens of a morphologically similar population from the Ko'olau Range on East O'ahu were examined (provided by D. Polhemus). The male specimens are dorsally paler than the *N. makaihe* (West O'ahu); the genitalia were not examined due to limited sample size. Eastern O'ahu populations may represent a potential sibling species system as seen on Maui and Hawai'i (Fig. 3). Due to the difficulty of collecting species from *B. arguta* on the eastern side of O'ahu, we are unable to make an adequate comparison of the two populations here.

Phylogenetic evidence places *N. makaihe* in a clade with the *kanawao* species group, with low support. *N. makaihe* is placed sister to a clade associated with the host plant genus *Myrsine* (Myrsinaceae), containing two species found on East Maui and Hawai'i island (COII % divergence avg. = 16.03%). Both taxa occur in high elevation (~914.4m) rainforest.

Further gene and taxonomic sampling are required to resolve this relationship. *Nesophrosyne makaihe*'s internal genitalia is considerably different, further confusing inference of relationships to other species associated with *B. arguta*.

Nesophrosyne magnaccai Bennett sp. nov.

(Fig. 6a-g)

Diagnosis:

Length: male = 4.7mm, female = 5.60mm. Brown species with conspicuous dark colored veins; without saddle mark or pale colored claval veins. Crown produced, bluntly rounded. Pygofer appearing triangular, with sharply produced apex rising at mid-length. Aedeagal arms widely splayed, appearing relatively compressed in ventral view; gonopore preapical, with apical processes recurving anterolaterad 1/5 length of aedeagal arms. Endemic to Moloka'i.

Description:

Dorsum Brownish species with forewing veins conspicuous (Fig. 6a). Crown predominately dark with thin pale line extending along posterior margin, triangular pale region emerging at apex. Pronotum almost entirely dark except for lateral edges, which are marked with two lateral pale spots on margins. Mesonotum dark. Scutellum central region variably dark, lateral margins pale. Forewing veins dark and conspicuous, including clavus; cells clear tinged with brown; central anteapical cell infused with dark pigmentation at ends; discal cells dark; lacking any pale coloration common in *Nesophrosyne*; outer anteapical cell present, triangular.
Venter: Face with clypeus, clypellus, lorum dark; gena dark with lateral margins pale. Abdominal segments almost entirely dark with thin pale line along each posterior margin; pleurites dark with posterior edge variably pale. Forelegs pale, base of sete dark; middle femora and tibia dark at joints; hind femora and tibia mostly dark with pale macrosete.
Genitalia: Pygofer (Fig. 6b) triangular, sharply produced at mid-height from base; dorsal and ventral edges narrowing straight to apex; ventral lobe produced; 11 macrosete. Aedeagus (Fig. 6f,c,g) comparatively compressed in posterior view, rising nearly above of central apodeme; aedeagal arms splayed widely; gonopore preapical; apical processes extending just above gonopore, hooking approximately 1/5 the length of aedeagal arms. Style (Fig. 6e) preapical lobe sloping, forming an oblique angular edge, with microsete. Connective (Fig. 6d) with posterior edge notched and wider than anterior arms; anterior arms thick, moderately splayed.

Distribution: USA: Hawaiian Islands, Moloka'i Eastern Mountain Range, >1340m, wet forest.

Measurements:

Body length (n=4): Male (n=4) = 4.70mm (4.46mm–4.80mm); Female (n=1) = 5.60mm.
Genitalia (n=3): Pygofer = 0.60mm (0.59mm–0.62mm); Style = 0.46mm (0.45mm–0.47mm); Connective = 0.32mm (0.32mm–0.33mm); Aedeagus lateral length = 0.39mm (0.38mm–0.39mm); Aedeagus posterior height = 0.13mm (0.13mm–0.14mm).

Material Examined:

Type material: Holotype: 1 male, Hawaiian Islands, Moloka'i, Kamakou Preserve, Pu'u Kolekole, N21^{o} 06.436 W156^{o} 54.141, Elevation: 1340m, 19Feb2007. Host Plant: *Broussaisia arguta*. Coll. K. Magnacca. Deposited in the BPBM, Honolulu, Hawai'i. Type #: 17303.
Additional material: 3 males, 1 female, same as holotype. Deposited in the BPBM, Honolulu, Hawai'i.

Etymology:

This species is named after its collector Dr. Karl Magnacca (Hawaiian Entomologist, University of Hawai'i, Hilo) for his contributions to this project and for his extensive, and helpful guidance in the field.

Discussion:

A single *Nesophrosyne* species associated with *Broussaisia arguta* is described from Moloka'i. Similarly to *N. heopoko*, the Moloka'i species is morphologically distinct from the other species described here. Current phylogenetic evidence provides weak support for an independent shift to *B. arguta*. *N. magnaccai* is placed sister to *N. sp.4* and *N. oblique* (data not shown), which are associated with the host plant genera *Lobelia* (Campanulaceae) and *Myrsine* (Myrsinaceae), respectively. Both sister taxa occur in on East Maui in high elevation (~914.4m) rainforest. COII percent divergence between *N. magnaccai* and *N. sp.4* avg. = 15.68%, and *N. magnaccai* and *N. obliqua* = 15.67%.

kanawao species group

(Figs. 7, 8a–g & 9a–g)

Diagnosis:

Dark species with conspicuous, pale coloration on clavus. Aedeagus with arms short, rising just above central apodeme in ventral view; gonopore apical; apical processes short and straight, extending 1/10 the length of the aedeagal arm. Connective distinct with anterior arms large, thickening at mid-length, appearing as 90^{o} angles along lateral edges. Endemic to Maui and Hawai'i Island.

Description:

Dorsum: Dark species with conspicuous pale coloration on clavus (Fig. 7). Mesonotum dark. Scutellum pale. Forewing veins obfuscate; costal cells hyaline, divided by dark pigmentation along R1; outer anteapical cell present, small and triangular. Clavus with extensive pale coloration, variable between species.
Venter: Clypeus with well formed grill mark on anterior half, posterior half forming dark basal triangle from which grill emerges; clypellus and lorum dark; gena dark with outer margins pale. Central abdominal segments dark with pale band expanding in anterior segments.
Genitalia: Aedeagus with relatively short arms rising just above central apodeme in ventral view; gonopore apical; apical processes short, extending anterolaterally 1/10 length of aedeagal arm (Figs. 8 & 9f,c,g); apical processes absent in *N. kaupoi*. Connective distinct with flat posterior edge, wider than waist; anterior arms thickening at mid-length, forming nearly 90^{o} angles along lateral edges; anterior ends curving slightly, hooked laterad (Figs. 8 & 9d).

Distribution: USA: Hawaiian Islands, Hawai'i Island and Maui, 910–2080m, wet forest (Fig. 3).

Etymology:

The *kanawao* species group is named after the Hawaiian word for *Broussaisia arguta* for the association of its constituent species with this plant species.

Discussion:

The *kanawao* species group is a monophyletic assemblage of species associated with *Broussaisia arguta* on Maui and Hawai'i Island (Fig. 3). We describe five species, further subdividing them into two species subgroups: *broussaisiai* species subgroup (endemic to Maui) and *aakokohaikea* species subgroup (endemic to Hawai'i Island). These delineations conform to species' morphological similarities, islands of endemicity, and monophyly (Fig. 3). Phylogenetically, the species subgroups are sister (COII % divergence avg. = 11.52%; Table 1), showing retention of the *B. arguta* host plant association through colonization of a novel island. Polarity of this biogeographic pattern requires more sampling to resolve.

Internal genitalia in the *broussaisiai* and *aakokohaikea* species subgroups show similarity in the placement and length of the apical processes, and the unique shape of the connective. External morphology easily distinguishes the subgroups (*e.g., aakokohaikea* species subgroup comprises darker species). Within subgroups, species are difficult to identify without knowledge of collection locality and subtle morphological differences, as there can be considerable overlap in external color patterning.

broussaisiai species subgroup

(Figs. 7a–c & 8a–g)

Diagnosis:

Dark species with conspicuous, highly variable pale coloration on clavus, ranging from entirely pale to a central oval saddle mark anteriorally flanked by pale blotches. Pronotum with posterior half pale. Pygofer with apex at 3/4 height from base; without basal lobe; dorsal edge depressed. Aedeagus with arms widening at mid length, showing an angular bend; apical processes absent in *N. kaupoi*. Endemic to Maui.

Description:

Dorsum: Dark species with conspicuous and variable pale coloration on clavus. Crown predominately black with variable central pale mark at apex and a thin pale line along posterior margin. Pronotum dark with posterior pale band up to 1/2 width. Mesonotum dark. Scutellum pale. Clavus with saddle mark ranging in pale pigmentation; darkest forms with only a central round saddle mark, usually anteriorally flanked by lateral pale marks variable in size and color; palest forms with clavus appearing entirely pale with posterior angles dark.
Venter: Pleurites largely pale with variable central dark patches. Legs almost entirely pale; hind tibiae dark along anteroventral edge.
Genitalia: Pygofer apex bluntly rounded and turned slightly dorsad, rising 3/4 height from base; without basal lobe; posterior-dorsal edge depressed from slight to completely concave; dorsal edge flat, tapering towards anterior end; 13–15 macrosete. Aedeagus with arms widening at mid-length, showing an angular bend in ventral view; apical processes absent in *N. kaupoi*. Style large; posterior hooks comparatively thick; preapical lobe, sloping towards anterior end, rest of medial lobe rounded; posterior arm towards connective curving mesad, 's' like. Connective anterior ends or arms curving, slightly hooked laterad.

Distribution: USA: Hawaiian Islands, Maui, 910m–2080m, wet forest (Fig. 3)

Etymology:

This species subgroup is named after the *Broussaisia* host plant genus.

Discussion:

We describe three sibling species from Maui occurring on *Broussaisia arguta*. Genetic evidence demonstrates unique, reciprocally monophyletic evolutionary lineages with relatively high sequence divergence occurring on West Maui, East Maui Haleakalā windward face, and East Maui Haleakalā leeward face (Kaupo Gap). COII percent sequence divergence for these geographic ranges are as follows (Table 1): Haleakalā windward face – Haleakalā leeward face = 11.24%; Haleakalā windward face – West Maui = 4.22%; and, Haleakalā leeward – West Maui = 10.57%.

Morphologically, these species can be difficult to distinguish, but some external characteristics combined with geographic information are adequate to separate them. In general, West Maui represents a darker form than the both East Maui species, overlapping with *N. ogradyi* variants. *N. kaupoi* overlaps in color with the paler variants of *N. ogradyi*. *Nesophrosyne kaupoi* occurs at higher elevation (~2080m) than the other described species (910m – 1220m), which corresponds to the upper elevation of *B. arguta*.

The geographic structuring of these species provides unique insight into the potential evolutionary mechanisms that may contribute to the diversification of the genus. Haleakalā leeward, due to its inhospitable geographic divide across Haleakalā crater, high elevation, or differences in precipitation (or a confluence of all), may contribute to the genetic isolation and speciation of *Nesophrosyne* in this range. The distance, precipitation change and elevation change going from West Maui to East Maui are sufficient to maintain isolation between these species.

Nesophrosyne broussaisiai Bennett sp. nov.

(Fig. 7a)

Diagnosis:

Length: male = 4.22mm, female = 5.21mm. A dark species, with three conspicuous pale spots on clavus. Clavus with a central round pale saddle mark, anteriorally flanked by lateral symmetrical pale marks, sometimes showing as clear patches along clavus (see Fig. 7a); darkest forms with only a central saddle mark. Crown and pronotum with posterior pale bands. Genitalia matching species group description (see Fig. 8b–g). Endemic to West Maui.

Distribution: USA: Hawaiian Islands, Maui, West Mauna Kahalawai, Pu'u Kukui, ~1220m, wet forest (Fig. 3).

Measurements:

Body length (n=5): Male (n=5) = 4.22mm (4.15mm–4.30mm); Female (n=8) = 5.21mm (5.10mm–5.40mm). ***Genitalia*** (n=5): Pygofer = 0.57mm (0.55mm–0.59mm); Style = 0.47mm (0.46mm–0.48mm); Connective = 0.28mm (0.27mm–0.29mm); Aedeagus lateral length = 0.35mm (0.34mm–0.37mm); Aedeagus posterior height = 0.15mm (0.14mm–0.16mm).

Material Examined:

Type Material: Holotype: 1 male, Hawaiian Islands, West Maui, Mauna Kahalawai, Pu'u Kukui, Boardwalk Trail, N20^{o} 56.078 W156^{o} 36.985, Elevation: 1220m, 23Nov2009. Host Plant: *Broussaisia arguta*. Coll. G.M. Bennett, K. Magnacca, P.M. O'Grady. Deposited in the BPBM, Honolulu, Hawai'i. Type #: 17304. ***Additional Material***: 12 males, 11 females, Hawaiian Islands, West Maui, Mauna Kahalawai, Pu'u Kukui, Boardwalk Trail, N20^{o} 56.078 W156^{o} 36.985, Elevation: 1220m, Elevation: 940m, 23Nov2009, 7Aug2007, Coll. G.M. Bennett, K. Magnacca, P.M. O'Grady. Deposited in the BPBM, Honolulu, Hawai'i. 1 female, Hawaiian Islands, West Maui, Wet Forest below Nakalalua, Pu'u Kukuii Trail, 1220m, 26May2004. N20^{o} 54'58" W156^{o} 35'37", Coll: DA Polhemus. Deposited in D.A. Polhemus's personal collection at the Smithsonian, Washington D.C.

Etymology:

The name chosen for this species refers to the host plant genus name it occurs on, *Broussaisia*.

Discussion:

This species is nearly indistinguishable from the East Maui sibling species. Morphologically, the only character that distinguishes *N. broussaisiai* is the variable color pattern on the clavus, which is generally darker.

Nesophrosyne ogradyi Bennett sp. nov.

(Fig. 8a–g)

Diagnosis:

Length: male = 4.17mm, female = 5.65mm. A paler species than *N. broussaisiai*. Clavus entirely pale or with three spots, with a central round saddle mark flanked by lateral pale marks (Fig. 8a). Pronotum posterior 1/2 pale. Genitalia matching species group description (Fig. 8b–g). Endemic to East Maui, Haleakalā windward face.

Distribution: USA: Hawaiian Islands, Maui, Haleakalā windward face, >910m, wet forest (Fig. 3)

Measurements:

Body length: Male (n=10) = 4.17mm (4.00mm–4.30mm); Female (n=10) = 5.65mm (5.50mm–5.80mm). ***Genitalia*** (n=5): Pygofer = 0.57mm (0.56mm–0.58mm); Style = 0.47mm (0.46mm–0.48mm); Connective = 0.27mm (0.26mm–0.28mm); Aedeagus lateral length = 0.31mm (0.30mm–0.33mm); Aedeagus posterior height = 0.16mm (0.15mm–0.17mm).

Material Examined:
Type Material: Holotype: 1 male, Hawaiian Islands, East Maui, Waikamoi Forest Reserve, Heed Trail, N20^{o} 48.638 W156^{o} 14.509, Elevation: 1310m, 31July2009. Host Plant: *Broussaisia arguta*. Coll. G.M. Bennett, P.M. O'Grady, K.M. Magnacca. Deposited in the BPBM, Honolulu, Hawai'i. Type #: 17305. ***Additional Material***: 13 males, 17 females, Hawaiian Islands, East Maui, Makawao Forest Preserve, N20^{o} 48.638 W156^{o} 14.509, Elevation: 1310m, 31July2007, 6Aug2007, 6June2009. Host Plant: *Broussaisia arguta*. Coll. G.M. Bennett. Deposited in the BPBM, Honolulu, Hawai'i. 2 males, 2 females, Hawaiian, Islands, East Maui, Waikamoi Forest Reserve, N20^{o} 48.397 W156^{o} 15.295, Elevation: 1300m, 1June2009. Host Plant: *Broussaisia arguta*. Coll. G.M. Bennett. P.M. O'Grady, K.M. Magnacca. Deposited in the BPBM, Honolulu, Hawai'i. 5 males, Hawaiian Islands, Maui, Haleakalā, Pu'u O Kakai, TNCH, Waikamoi Preserve, N20^{o} 48'00" W156^{o} 14'44", Elevation: 1500m, 16May2003. Host Plant: *Broussaisia arguta*. Coll: D.A. Polhemus. Deposited in D.A. Polhemus's personal collection at the Smithsonian, Washington D.C.

Etymology:

This species is named after Dr. Patrick O'Grady (Hawaiian Dipterologist) for his invaluable contributions to this project and for his positive scientific mentorship of G.M. Bennett.

Discussion:

Nesophrosyne ogradyi represents a lighter form than *N. broussaisiai*. External morphology is nearly identical to *N. kaupoi*. *Nesophrosyne kaupoi* is distinguishable from *N. ogradyi* by its absent apical processes.

Nesophrosyne kaupoi Bennett sp. nov.

(Fig. 7c)

Diagnosis:

Length: male = 4.18mm, female = 5.75mm. Paler species, matching palest forms of *N. ogradyi*. Crown with pale blotches. Clavus entirely pale except for posterior 1/4, which forms anterior dark triangles. Aedeagus without apical processes. Endemic to Maui, Haleakalā leeward face, Kaupo Gap.

Measurements:

Body length: Male (n=3) = 4.18mm (4.10mm–4.25mm); Female (n=4) = 5.75mm (5.70mm–5.80mm). ***Genitalia*** (n=2): Pygofer = 0.58mm (0.57mm–0.59mm); Style = 0.48mm (0.47mm–0.48mm); Connective = 0.29mm (0.28mm–0.29mm); Aedeagus lateral length = 0.33mm (0.32mm–0.34mm); Aedeagus posterior height = 0.15mm (0.13mm–0.16mm).

Material examined:

Type Material: Holotype: 1 male Hawaiian Islands, East Maui, Haleakalā, Paliku, Ridge Trail, N20^{o} 43.074 W156^{o} 08.470, Elevation: 2070m, 1Aug2007. Host Plant: *Broussaisia arguta*. Coll. G.M. Bennett, P.M. O'Grady, R. Lapoint, K.M. Magnacca. Deposited in the BPBM, Honolulu, Hawai'i. Type #: 17306. ***Additional Material***: 2 males, 1 female, same as holotype. Deposited in the BPBM, Honolulu, Hawai'i. 2 males, 3 females, Hawai'i Islands, East Maui, Paliku Seep, N20^{o} 43.074, W156^{o} 08.470, Elevation: 2070m, 1Sept2007. Host Plant *Broussaisia arguta*. Coll: K Magnacca. Deposited in the BPBM, Honolulu, Hawai'i.

Etymology:

This species is named after its collection locality and area of endemicity.

Discussion:

Nesophrosyne kaupoi occurs at a significantly higher elevation than the other *broussaisia* subgroup species. It is the only species in the species group missing the apical processes on the aedeagal arms.

aakokohaikea species subgroup

(Figs. 7d–e & 9)

Diagnosis:

Dark species, with pale coloration restricted to claval veins, scutellum, and posterior edge of crown. Pygofer apex bluntly pointed, rising at 1/3 height from base; basal lobe present, reduced. Aedeagal arms without thickening angular bend. Style with elongate, hooked thin posterior processes; preapical lobe flat. Endemic to Hawai'i Island.

Description:

Dorsum: Dark species, appearing almost entirely black except for articulated pale pigmentation along claval veins. Crown dark, with thin pale line along length of posterior margin (see Figs. 7d–e & 9a). Pronotum and mesonotum dark. Scutellum pale, usually with longitudinal dark line extending to posterior apex. Forewings predominately dark, with veins and cells mostly obfuscate. Clavus with veins irregularly pale, often with disjunct pigmentation at anterior intersection of claval veins; darkest forms with pale veins extending form commissural line as

two thin lines curved anterad; palest species with pale pigmentation bleeding throughout clavus from center, appearing predominately pale.
Venter: Hind femora and base of tarsal segments dark, rest pale.
***Genitalia*:** Pygofer (Fig. 9b) bluntly pointed at 1/3 height from base; ventral lobe reduced, edge flat and angled posteroventrad; dorsal edge flat; 12 macrosete. Aedeagus (Fig. 9c,f,g) with apodeme appearing pointed; aedeagal arms splayed widely. Style (Fig. 9e) with thin posterior hook approximately 1/3 length of style; preapical lobe flat with microsete, slightly slanted; medial lobe with nearly straight edge extending towards posterior processes; microsete present. Connective (Fig. 9d) anterior ends of arms straight and pointed.

Distribution: USA: Hawaiian Islands, Hawai'i, ~820m–1520m, wet forest (see Fig. 3).

Etymology:

The name chosen for this species subgroup is adopted from one of its constituent species.

Discussion:

The *aakokohaikea* species subgroup is closely related to the *broussaisia* species subgroup endemic to Maui (Fig. 3), showing genetic sub-structuring according to volcanic mountains similar to the Maui species (COII % divergence avg. = 9.23%; Table 1). The Hawai'i Island sibling species are difficult to discern morphologically, as no consistent characters differentiate the lineages, except for subtle color variation on the clavus.

Sampling for this study is limited to two geographic ranges or volcanic mountains: Kohala Range and Mauna Loa. Further investigation of other mountain ranges (*e.g.,* Mauna Kea and Hualālai) would undoubtedly reveal other sibling species to be placed in the *aakokohaikea* species subgroup.

Nesophrosyne aakokohaikea Bennett sp. nov.

(Fig. 9a–g)

Diagnosis:

Length: male = 4.06mm, female = 5.51mm. Dark species with claval veins variably pale; darkest forms show predominately pale veins with pigmentation infused throughout the surrounding area; palest forms small length of claval veins pale extending from commissural line (Fig. 9a). Endemic to Hawai'i Island, Mauna Loa South Slope.

Distribution: USA: Hawaiian Islands, Hawai'i, South Eastern Slope of Mauna Loa, ~820m–1160m, wet forest (Fig. 3).

Measurements:

Body length: Male (n=6) = 4.06mm (3.92mm – 4.12mm); Female (n=10) = 5.51mm (5.3mm–5.8mm). ***Genitalia*** (n=6): Pygofer = 0.56mm (.54mm–0.57mm); Style = 0.45mm (0.44mm–

0.47mm); Connective = 0.26mm (0.24mm–0.27mm); Aedeagus lateral length = 0.33mm (0.32mm–0.35mm); Aedeagus posterior height = 0.14mm (0.13mm–0.16mm).

Material examined:

Type Material: Holotype: 1 male, Hawaiian Islands, Hawai'i, Ka'u Forest Reserve, Kuahiwi Ranch, N19^{o} 05.111 W155^{o} 35.830, Elevation: 820m, 15Jan2009. Host Plant: *Broussaisia arguta*. Coll. G.M. Bennett. Deposited in the BPBM, Honolulu, Hawai'i. Type #: 17308. ***Additional Material***: 1male, Hawaiian Islands, Hawai'i, Hawai'i Volcanoes National Park, Ōla'a Forest, N19^{o} 27.725 W155^{o} 14.875, Elevation: 1170m, 7January2009. Host Plant: *Broussaisia arguta*. Coll. G.M. Bennett. Deposited in the BPBM, Honolulu, Hawai'i. 4 males, 1 female, Hawaiian Islands, Hawai'i, Hawai'i Volcanoes National Park, Ka'u, Kahuku Ranch, N19^{o} 06.0654 W155^{o} 40.582, Elevation: 1070m, 13Jan2009. Host Plant: *Broussaisia arguta*. Coll. G.M. Bennett. Deposited in the BPBM, Honolulu, Hawai'i. 3 males, 1 female, Hawaiian Islands, Hawai'i, Ka'u Forest Reserve, Kuahiwi Ranch, N19^{o} 05.111 W155^{o} 35.830, Elevation: 840m, 15Jan2009. Host Plant: *Broussaisia arguta*. Coll. G.M. Bennett. Deposited in the BPBM, Honolulu, Hawai'i. 21 males, 10 female, Hawaiian Islands, Hawai'i', Ka'u District, Mountain House Road, N19^{o} 09'34" W155^{o} 37'10", Elevation: 3494m, 24May2001. Host Plant: *Broussaisia arguta*. Coll: DA Polhemus. Deposited in D.A. Polhemus's personal collection at the Smithsonian, Washington D.C. 3 male, Hawaiian Islands, Hawai'i, Kiluea "29 miles", 1220m, 29Sept1917. Host Plant: Unknown. Coll: WM. Giffard. Deposited in D.A. Polhemus's personal collection at the Smithsonian, Washington D.C.

Etymology:

The name chosen for this species is Hawaiian for pale vein: *Aakoko* is the noun for vein, and *Haikea* is the adjective for pale. The name was chosen for the pale coloration along the claval veins.

Discussion:

Nesophrosyne aakokohaikea is difficult, and in some cases cannot be, morphologically separated from *N. kanawao* despite relatively high sequence divergence (Table 1). Knowledge of collection locality is necessary to differentiate the species without genetic information. Percent COII sequence divergence between species from Ka'u (western edge of range) and Ōla'a (eastern edge of range) is approximately 2%. This suggests that there may be some limitation of dispersal across this range.

Nesophrosyne kanawao Bennett sp. nov.

(Fig. 7d)

Diagnosis:

Length: male = 4.19mm, female = 5.45mm. Dark species. Clavus predominately and irregularly pale, pigmentation bleeding throughout clavus from claval veins; costal cells and proximal cells partly hyaline (Fig. 7d). Endemic to Kohala Mountain Range, Hawai'i Island.

Distribution: USA: Hawaiian Islands, Hawai'i, North West Kohala Mountain Range, 1520m, wet forest (Fig. 3).

Measurements:

Body length (n=4): Male (n=4) = 4.19mm (4.17mm – 4.21mm); Female (n= 4) = 5.45mm (5.3mm–5.6mm). ***Genitalia*** (n=4): Pygofer = 0.56mm (0.55mm–0.57mm); Style = 0.48mm (0.48mm–0.49mm); Connective = 0.27mm (0.26mm–0.28mm); Aedeagus lateral length = 0.36mm (0.34mm–0.37mm); Aedeagus posterior height = 0.22mm (0.21mm–0.23mm).

Material Examined:

Type Material: Holotype: 1 male, Hawaiian Islands, Hawai'i, Kohala Mountains, Kawaihae Oka, Pu'u O Umi Natural Area Reserve, Elevation: 1520m, N20^{o} 04.947 W155^{o} 45.128, 5October2006. Host Plant: *Broussaisia arguta*. Coll. G.M. Bennett. Deposited in the BPBM, Honolulu, Hawai'i. Type #: 17307. ***Additional Material***: 5 males, 4 females, same as holotype. Deposited in the BPBM, Honolulu, Hawai'i.

Etymology:

The *kanawao* species group is named after the Hawaiian word for *Broussaisia arguta* for the association of its constituent species with this plant species.

Discussion:

There is considerable overlap in the external coloration between *N. kanawao* and *N. aakokohaikea*, despite relatively large COII percent divergence (COII % divergence avg. = 9.23%). *Nesophrosyne kanawao* occurs in the Kohala Range, which is higher in elevation than the described *N. aakokohaikea*. Similar to species in the *broussaisia* species subgroup, it is unknown if adaptation to elevational gradients or geographic limits to dispersal contribute to speciation in this subgroup. Sampling from across Hawai'i Island on different volcanoes and at varying elevations would help elucidate this question.

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Table 1: Uncorrected pairwise distances for newly described *Nesophrosyne* species associated with *Broussaisia arguta*.

		Hawai'i		**Maui**		
		Kohala	Mauna Loa South Slope	West Maui	Haleakala Crater, E. Maui	Haleakala North Slope, E. Maui
Hawai'i	Kohala	0.0014				
	Mauna Loa South Slope	0.0923	0.0182			
Maui	West Maui	0.1067	0.1152	0.0077		
	Haleakala Crater, E. Maui	0.0990	0.1282	0.1057	0	
	Haleakala North Slope, E. Maui	0.1153	0.1269	0.0422	0.1124	0.0049

Figure 1: *Nesophrosyne* wing venation illustrating the variable outer anteapical cell. A) *Nesophrosyne (Nesophrosyne) perkinsi*, and B) *Nesophrosyne (Nesoreias) insularis*. Adapted from Kirkaldy (1948).

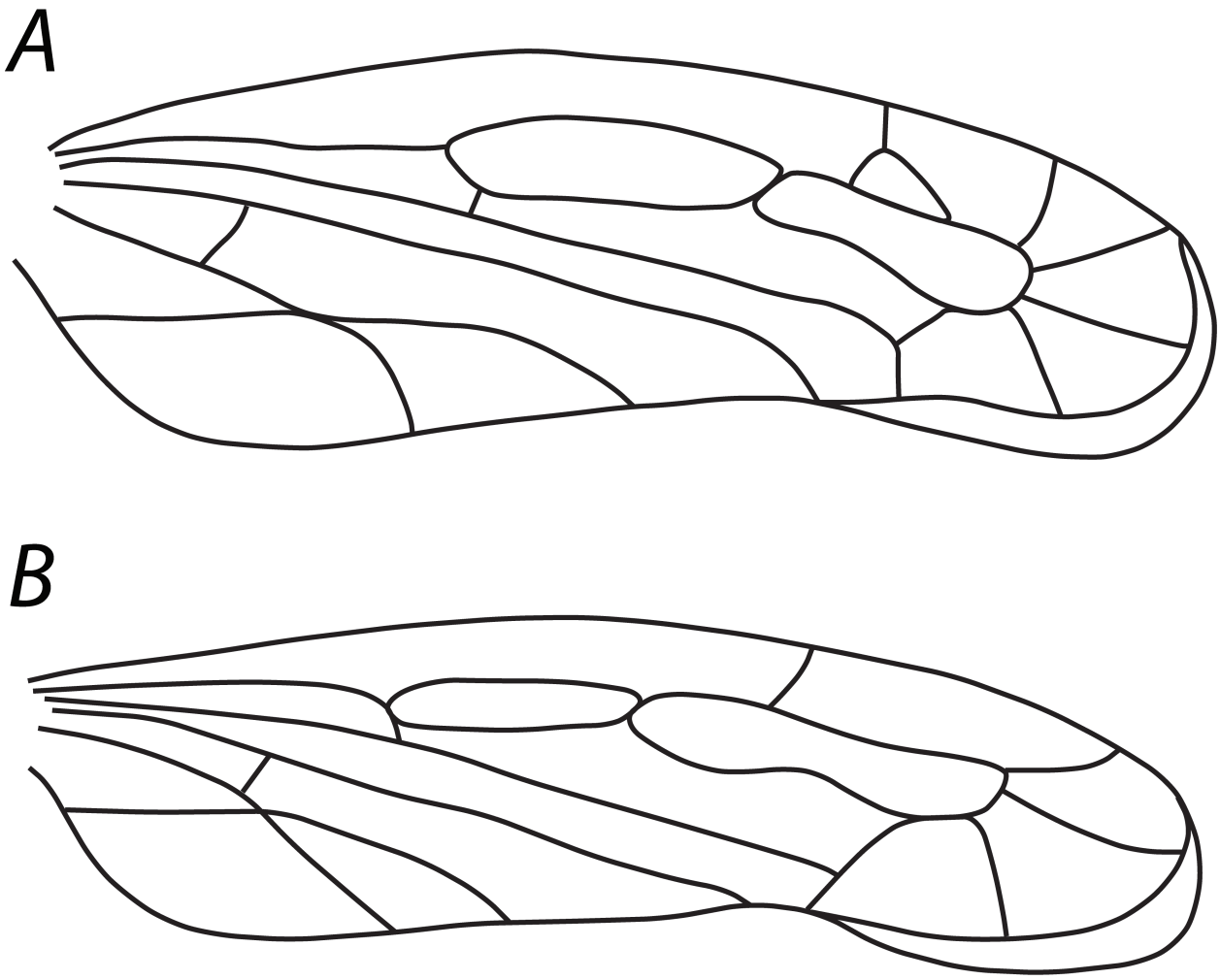


Figure 2: Hawaiian Islands. Boxes show per island estimates of the current number of species (*Current Species Number*) and the number of species previously described (*Previous Species Number*; Kirkaldy 1907, 1910; Osborn 1935; Zimmerman 1948).

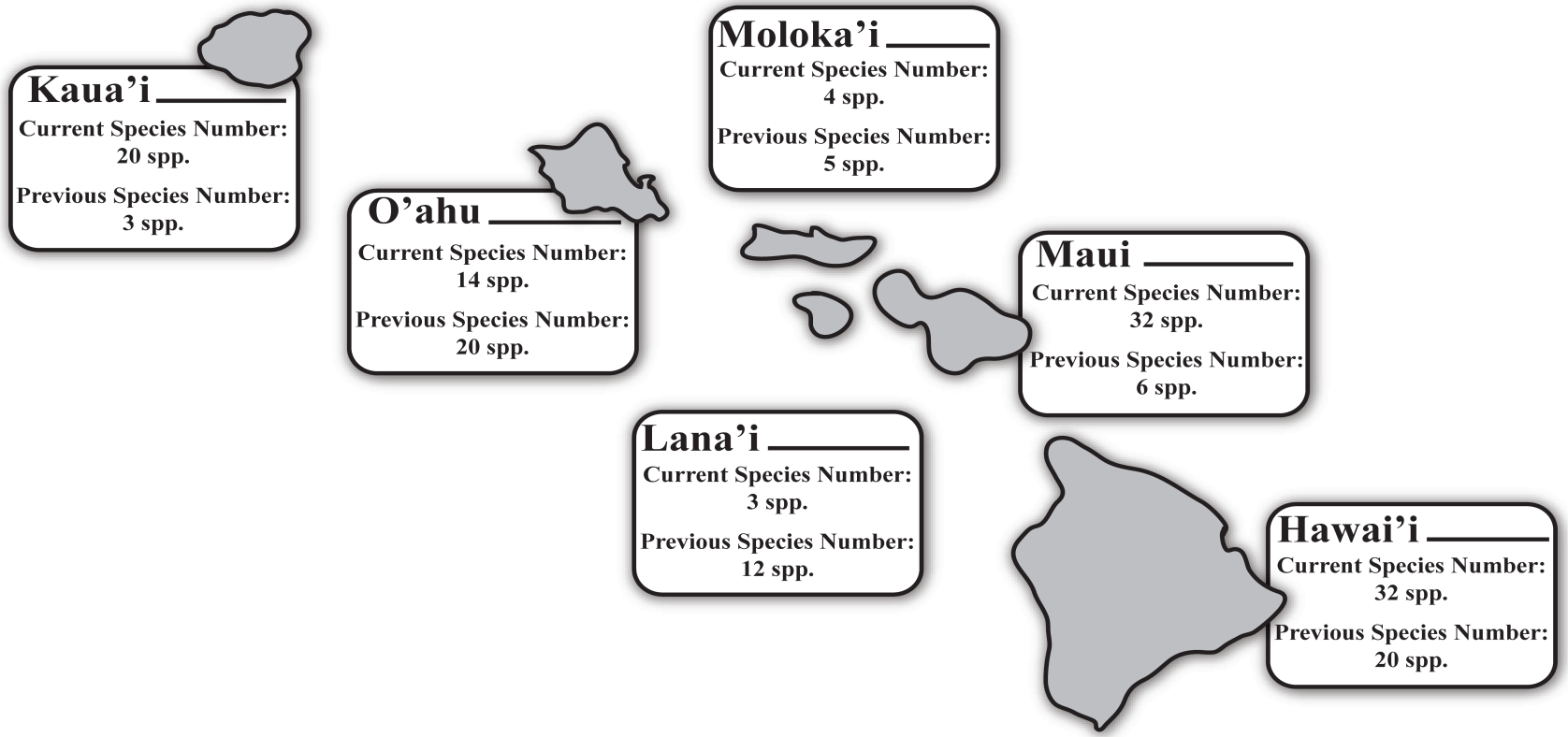

Kaua'i
Current Species Number:
20 spp.
Previous Species Number:
3 spp.
O'ahu
Current Species Number:
14 spp.
Previous Species Number:
20 spp.
Moloka'i
Current Species Number:
4 spp.
Previous Species Number:
5 spp.
Maui
Current Species Number:
32 spp.
Previous Species Number:
6 spp.
Lana'i
Current Species Number:
3 spp.
Previous Species Number:
12 spp.
Hawai'i
Current Species Number:
32 spp.
Previous Species Number:
20 spp.

Figure 3: Partitioned Maximum Likelihood phylogeny of *Nesophrosyne* (Hemiptera: Cicadellidae) species reconstructed in RAxML-VI-HPC v7.2.5 under a GTR+I+Γ likelihood model with 100 bootstrap partitions. Gene partitions are according to the mitochondrial genome (COII, 16s) and nuclear genome (H3, Wingless). Subsections A & B highlight species relationships of newly described species occurring on the host plant *Broussaisia arguta*. A) Silhouette of full Maximum Likelihood phylogeny results. B) *Nesophrosyne heopoko's* associated with other species on Kaua'i. C) Relationships of the species within the *kanawao* species group on Maui and Hawai'i. The numbers above branches are bootstrap support values.

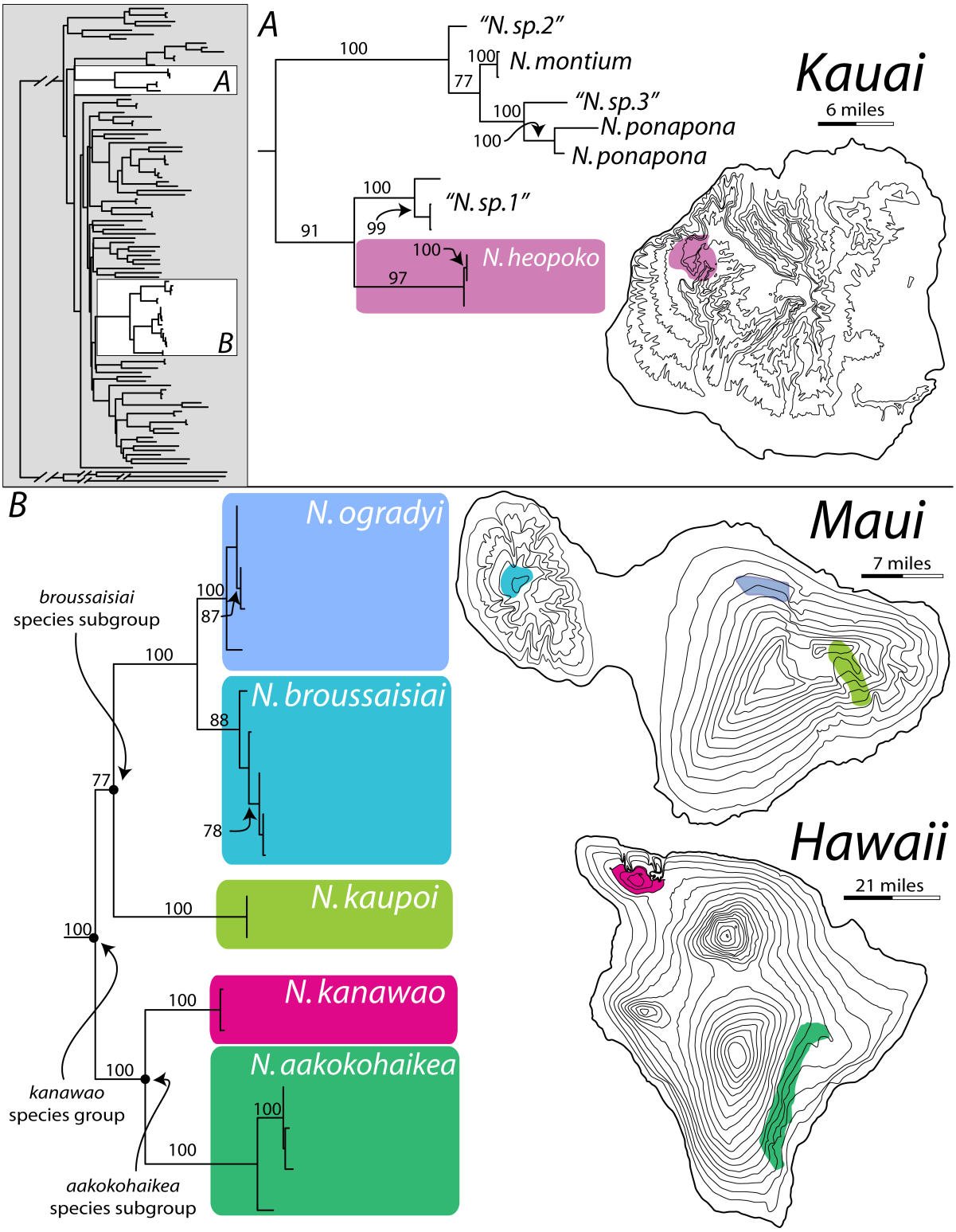

A
B
A
"N. sp.2"
N. montium
"N. sp.3"
N. ponapona
N. ponapona
"N. sp.1"
N. heopoko
100
100
77
100
100
100
91
99
100
97
Kauai
6 miles
B
N. ogradyi
broussaisiai
species subgroup
N. broussaisiai
N. kaupoi
N. kanawao
N. aakokohaikea
kanawao
species group
aakokohaikea
species subgroup
100
87
100
88
78
77
100
100
100
100
100
100
Maui
7 miles
Hawaii
21 miles

Figure 4: *Nesophrosyne heopoko* endemic to Kaua'i: a. dorsum; b. male pygofer; c. aedeagus ventral view; d. connective; e. style; f. aedeagus posterior view; and, g. aedeagus lateral view.

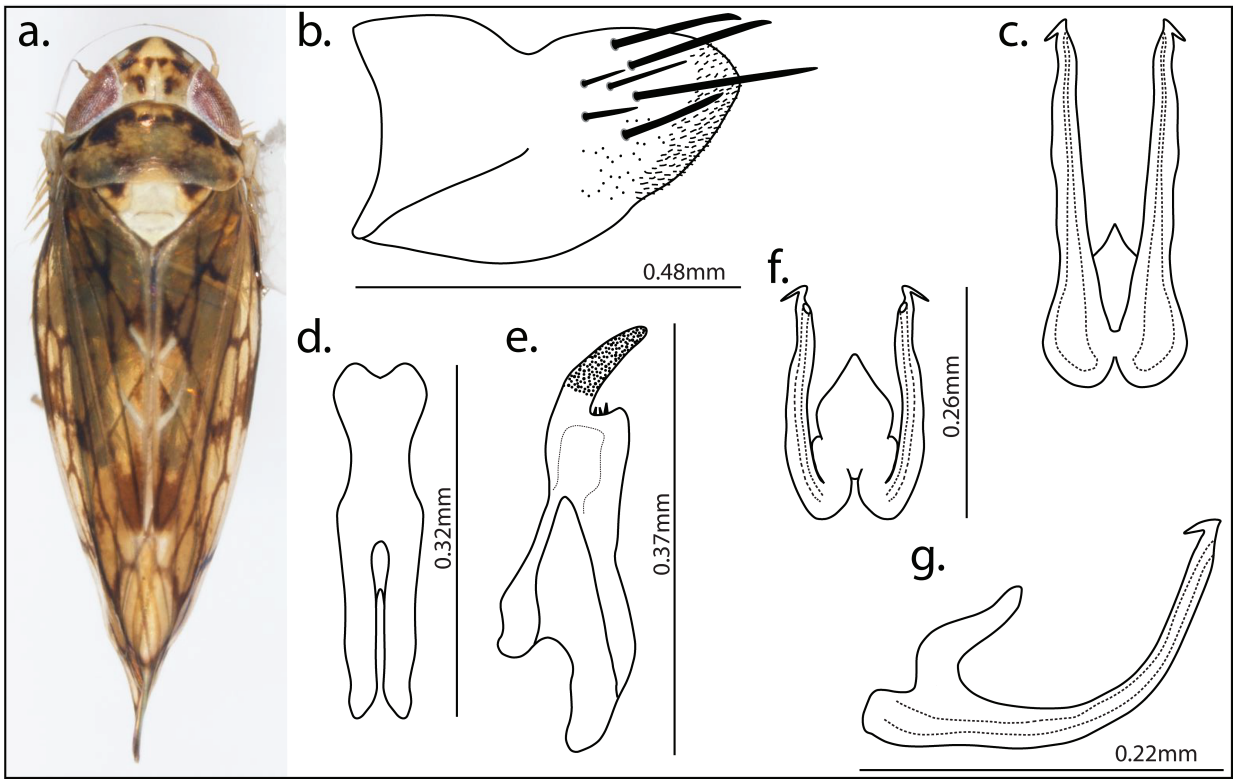


Figure 5: *Nesophrosyne makaihe* endemic O'ahu, Wai'anae Range: a. dorsum; b. male pygofer; c. aedeagus ventral view; d. connective; e. style; f. aedeagus posterior view; and, g. aedeagus lateral view.

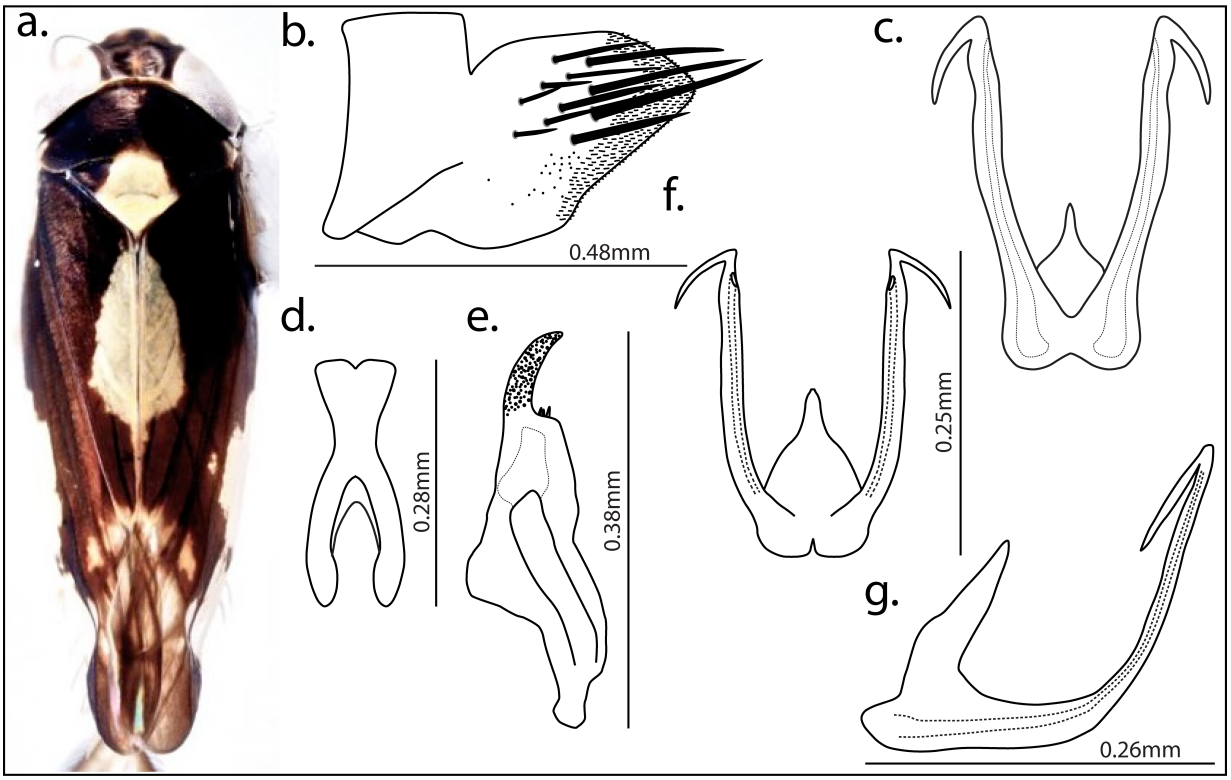


Figure 6: *Nesophrosyne magnaccai* endemic to Moloka'i: a. dorsum; b. male pygofer; c. aedeagus ventral view; d. connective; e. style; f. aedeagus posterior view; and, g. aedeagus lateral view.

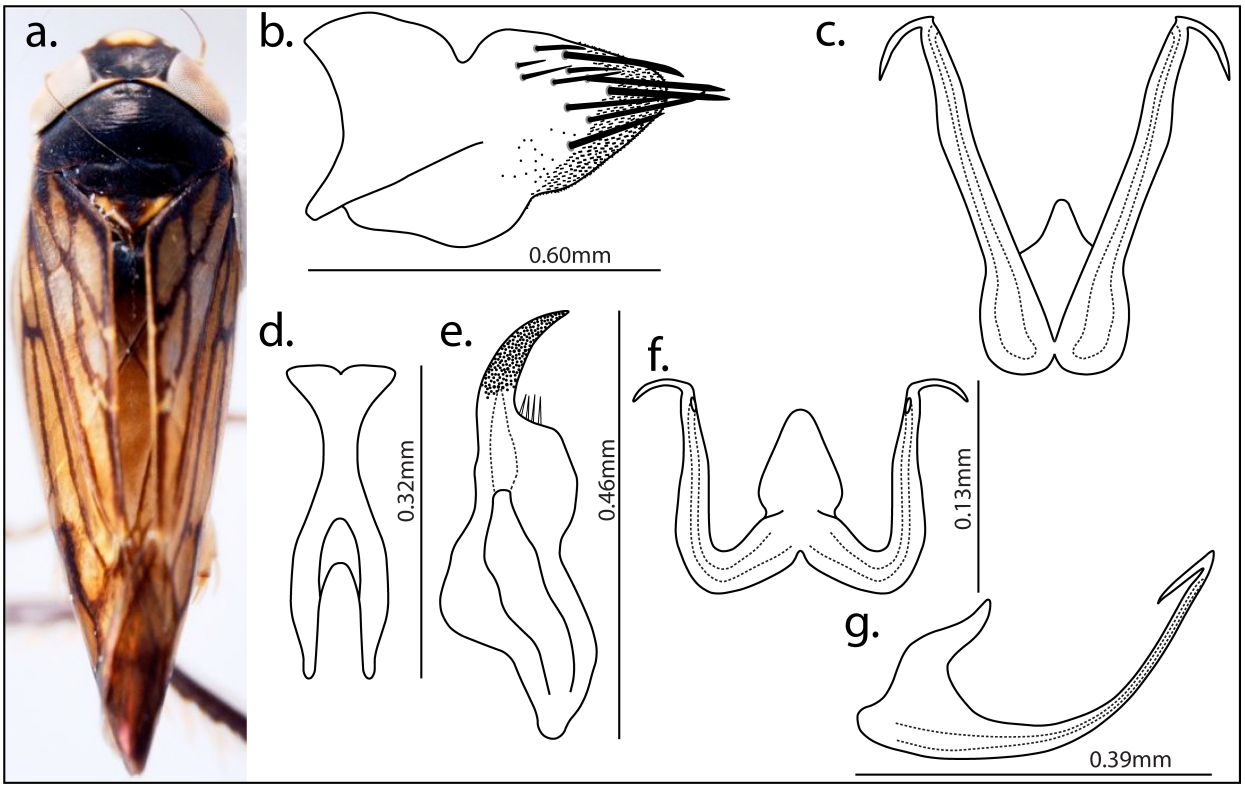


Figure 7: Dorsal habitus of the species comprising the *kanawao* species group: A. *N. broussaisiai*, B. *N. ogradyi*, C. *N. kaupoi*, D. *N. kanawao*, and E. *N. aakokohaikea*.

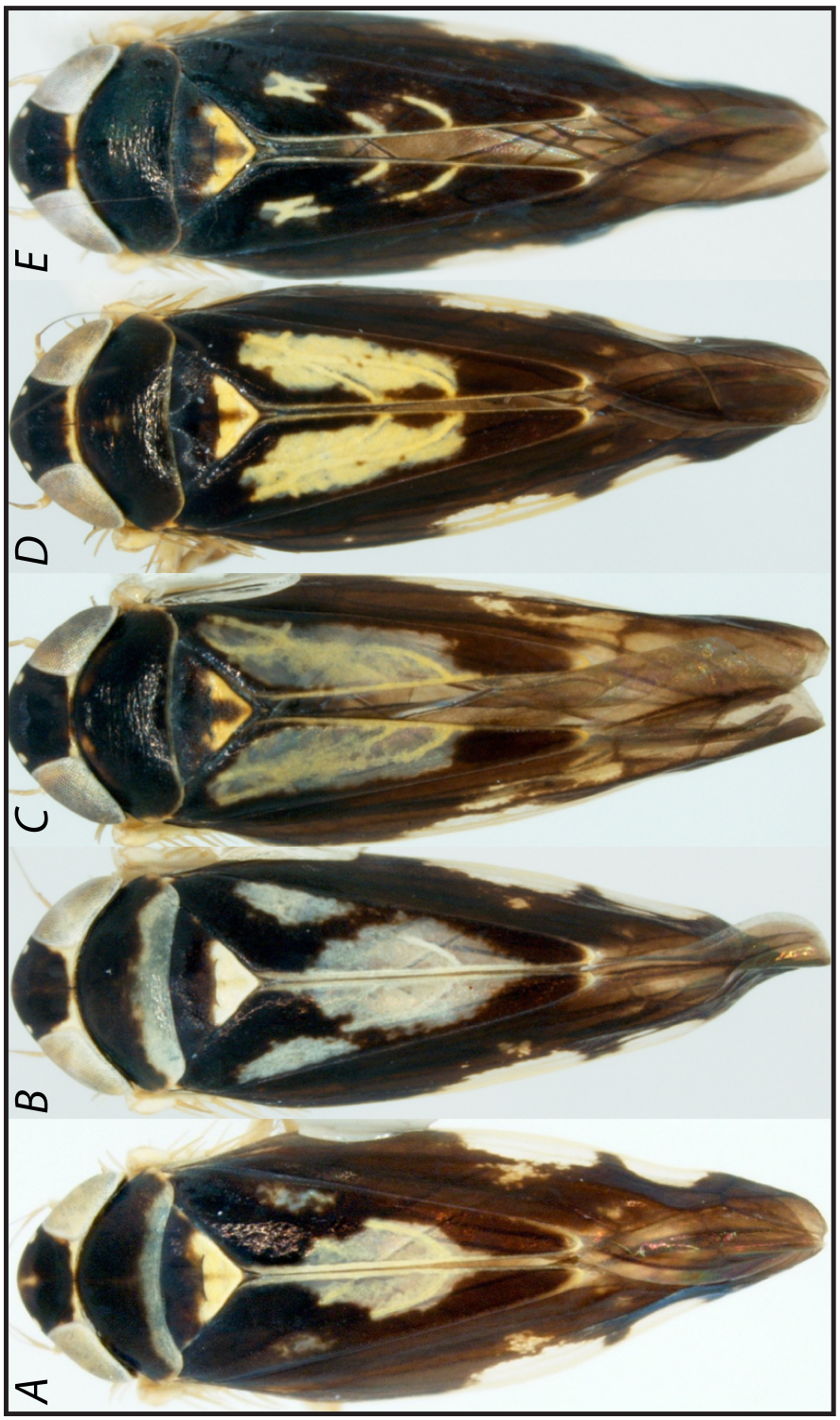


Figure 8: *Nesophrosyne ogradyi* endemic to Maui, Haleakalā windward face: a. dorsum; b. male pygofer; c. aedeagus ventral view; d. connective; e. style; f. aedeagus posterior view; and, g. aedeagus lateral view.

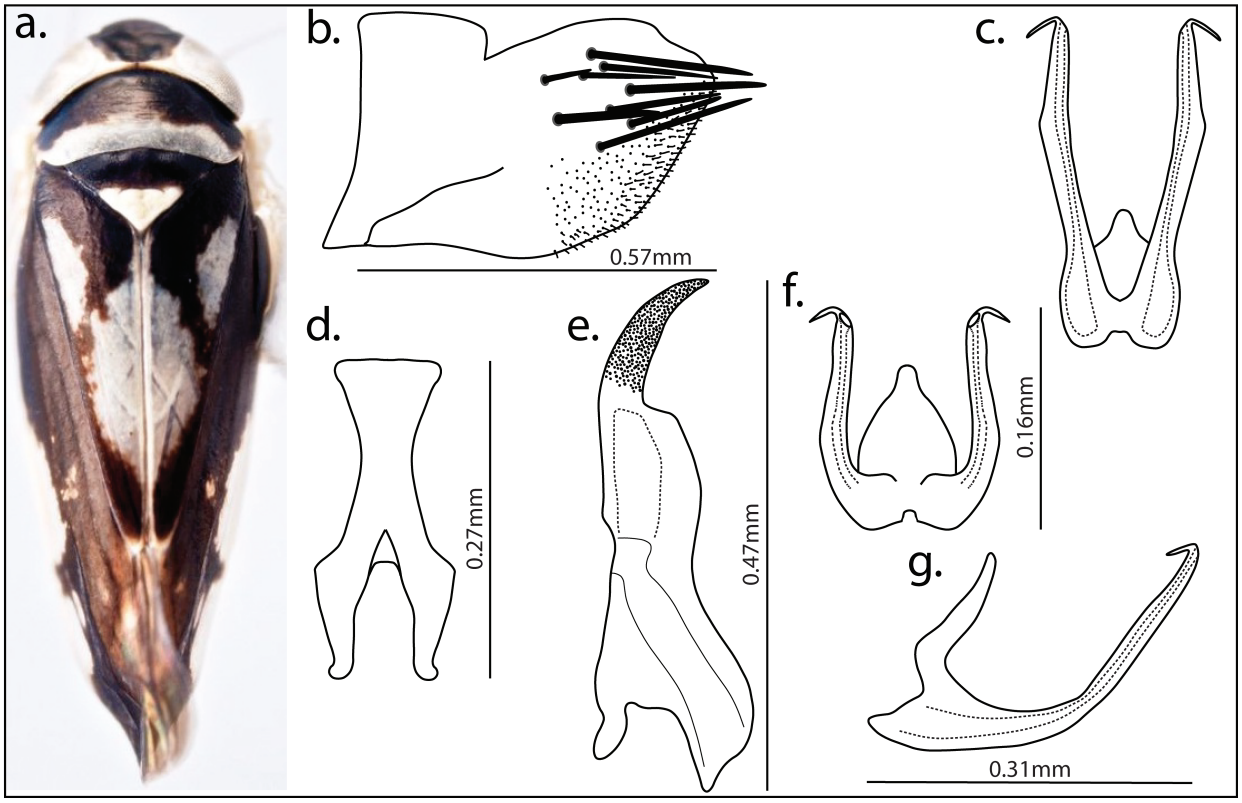
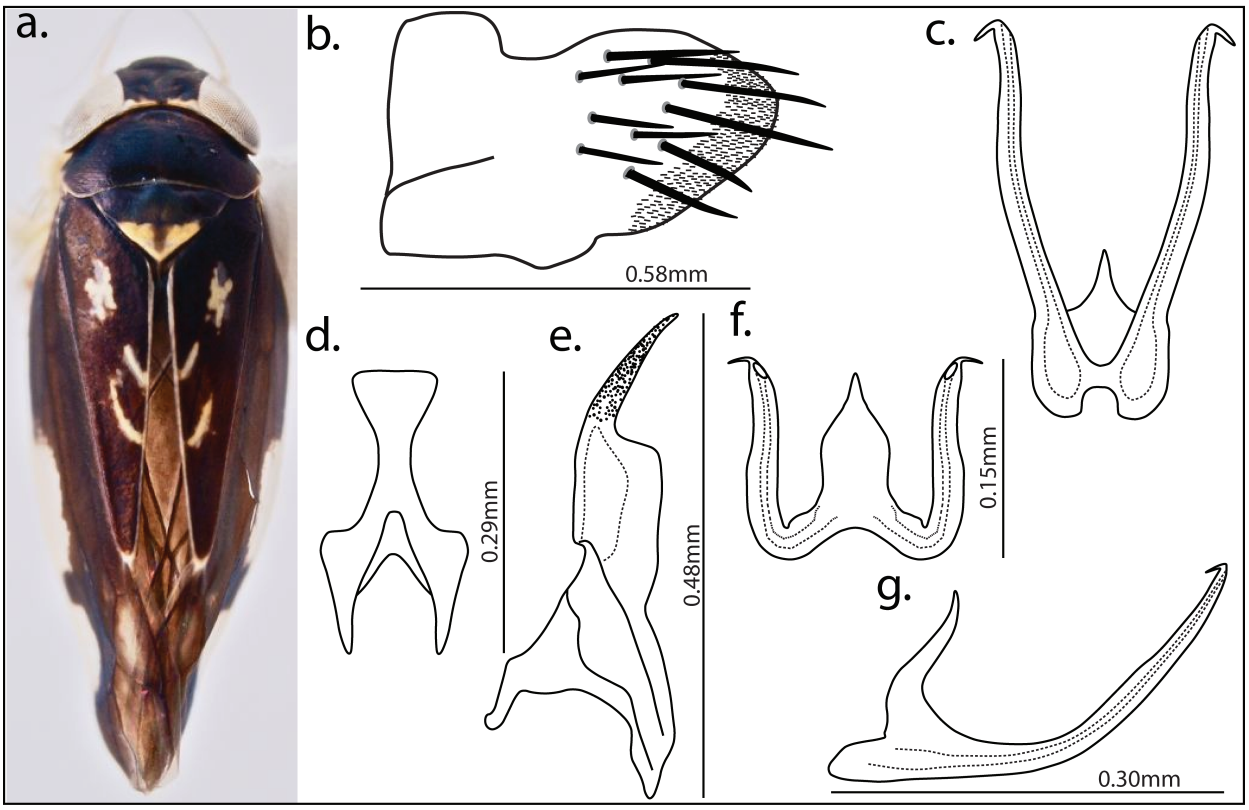


Figure 9: *Nesophrosyne aakokohaikea* endemic to Hawai'i Island, Mauna Loa South Slope: a. dorsum; b. male pygofer; c. aedeagus ventral view; d. connective; e. style; f. aedeagus posterior view; and, g. aedeagus lateral view.



Appendix 1: Species list of material examined for subgeneric classification and examination of outer anteapical cell character state distribution. Undescribed species are given provisional names. Undetermined species are given collection identification numbers.

Subgenus	**Species**	**Island**	**Notes on outer anteapical cell**
Nesoreias			
	N. eburneola	Hawai'i	Absent
	N. insularis	Hawai'i	Absent
	N. marginalis	Hawai'i	Absent
Nesophrosyne			
	N. umbratilis	Kaua'i	Present
	N. heopoko	Kaua'i	Present
	N. monticola	O'ahu	Present
	N. pelea	O'ahu	Present
	N. maritima	O'ahu	Present, extremely variable in shape and size. Inner anteapical cell sometimes subdivided
	N. makaihe	O'ahu	Present
	N. bobea	O'ahu	Present
	N. ponapona	O'ahu	Completely absent in some individuals
	N. procellaris	Moloka'i	Present. Additional cells adjacent to inner anteapical cell
	N. magnaccai	Moloka'i	Present
	N. lineata	Lana'i	Present, large
	N neuneu	Maui	Sometimes absent. Variable in presence, shape and size
	N. haleakala	Maui	Present, subdivided in some individuals
	N. obliqua	Maui	Present
	N. ogradyi	Maui	Present, reduced and absent in one wing for some individuals
	N. broussaisiai	Maui	Present, variable in size
	N. kaupoi	Maui	Present
	N. anguilifera	Maui	Present
	N. craterigena	Hawai'i	Sometimes absent. Variable in presence, shape and size
	N. caelicola	Hawai'i	Present
	N. cinera	Hawai'i	Present, large and variable in shape
	N. giffardi	Hawai'i	Present, small

	N. pele	Hawai'i	Present
	N. pluvialis	Hawai'i	Sometimes absent. Variable in presence, shape and size
	N. kanawao	Hawai'i	Present
	N. mabae	Hawai'i	Present
	N. giffardi interrupta	Hawai'i	Present
	N. aakokohaikea	Hawai'i	Present
Undescribed			
	"N. mamaki"	Kaua'i	Absent in all individuals
	"N. scaevolai"	Kaua'i	Sometimes absent from one wing.
	"N. clermontiai"	Kaua'i	Present, extremely large
	"N. nualolo"	Kaua'i	Present
	"N. solaris"	Kaua'i	Present, variable in size
	"N. rutilus"	Kaua'i	Missing entirely of in one wing
	"N. corcinus"	Kaua'i	Present
	"N. urerai"	Kaua'i	Present
	"N. ohiai"	O'ahu	Present
	"N. myrsinei"	O'ahu	Present
	"N. coprosmai"	O'ahu	Present
	"N. n.sp." (GB-17.1)	Moloka'i	Present
	"N. pequena"	Moloka'i	Absent in most individuals
	"N. n.sp" (GB-37.B)	Maui	Present, variable in size
	"N. lobeliai"	Maui	Present
	"N. pilo"	Maui	Sometimes absent. Variable in presence, shape and size
	"N. mimicaaffinis"	Maui	Sometimes absent. Variable in presence, shape and size
	"N. n.sp." (GB-034.D)	Maui	Present
	"N. montis"	Maui	Present
	"N. n.sp" (PO-389.6)	Maui	Present, large
	"N. giffardimaui"	Maui	Present
	"N. repagula"	Maui	Absent in one wing for some individuals
	"N. oliko"	Maui	Sometimes absent. Variable in presence, shape and size between wings
	"N. palevein"	Maui	Present
	"N. n.sp." (GB-35.6)	Maui	Present

	"N. pele-like"	Maui	Present
	"N. n.sp." (GB-35.A)	Maui	Present, subdivided in some individuals.
	"N. n.sp." (GB-34.E)	Maui	Present
	"N. atratus"	Maui	Present, variable in shape between wings.
	"N. trispot"	Maui	Present
	"N. palecalvus"	Maui	Present
	"N. po"	Maui	Present, elongate and subdivided in some individuals
	"N. n.sp" (GB-36.2)	Maui	Absent in one wing in most individuals
	"N. pipturihawaii"	Hawai'i	Present
	"N. n.sp." (GB-12.2)	Hawai'i	Present
	"N. n.sp." (GB-10.6)	Hawai'i	Present
	"N. n.sp." (GB-012.3)	Hawai'i	Present
	"N. n.sp." (GB-011.6)	Hawai'i	Present, variable in size and shape
	"N. n.sp." (GB-012.3)	Hawai'i	Present, extremely reduced
	"N. n.sp." (GB-010.D)	Hawai'i	Present
Undetermined			
	N. sp. (GB-021.1)	Kaua'i	Sometimes absent. Variable in presence, shape and size
	N. sp. (GB-022.4)	Kaua'i	Present, reduced
	N. sp. (GB-024.6)	Kaua'i	Present, extremely large, fusing with wing margin
	N. sp. (GB-029.2)	Kaua'i	Sometimes absent from one wing
	N. sp. (GB-029.1)	Kaua'i	Present but variable in size in all individuals
	N. sp. (km03)	Kaua'i	Missing in one wing for some individuals
	N. sp. (GB-030.5)	O'ahu	Present in all individuals
	N. sp. (GB-030.9)	O'ahu	Sometimes absent. Variable in presence, shape and size
	N. sp. (GB-031.7)	O'ahu	Present
	N. sp. (km32)	O'ahu	Present
	N. sp. (km33)	O'ahu	Present
	N. sp. (km34)	O'ahu	Sometimes completely absent, or missing in one wing
	N. sp. (GB-033.1)	Lana'i	Present, sometimes subdivided
	N. sp. (GB-033.4)	Lana'i	Present

	N. sp. (GB-034.1)	Maui	Present
	N. sp. (GB-034.A)	Maui	Present
	N. sp. (GB-035.6)	Maui	Present
	N. sp. (GB-035.8)	Maui	Present
	N. sp. 37	Hawai'i	Present
	N. sp. (GB-001.C)	Hawai'i	Present
	N. sp. (GB-002.1)	Hawai'i	Present, variable in size between wings
	N. sp. (GB-002.7)	Hawai'i	Present, reduced nearly absent
	N. sp. (GB-004.1)	Hawai'i	Present
	N. sp. (GB-005.1)	Hawai'i	Present, reduced
	N. sp. (GB-010.1)	Hawai'i	Sometimes absent. Variable in presence, shape and size
	N. sp. (GB-011.2)	Hawai'i	Present
	N. sp. (GB-016.1)	Hawai'i	Present
	N. sp. (GB-016.3)	Hawai'i	Present, elongate. Inner anteapical cell sometimes subdivided
	N. sp. (GB-011.5)	Hawai'i	Present
	N. sp. (GB-010.A)	Hawai'i	Present

CHAPTER 2

Host-Plants Shape Insect Diversity: Phylogeny, Origin, and Species Diversity of Native Hawaiian Leafhoppers (Cicadellidae: *Nesophrosyne*)

Abstract

Herbivorous insects and the plants on which they specialize, represent the most abundant terrestrial life on earth, yet their inter-specific interactions in promoting species diversification remains unclear. This study utilizes the discreet geologic attributes of Hawaii and one of the most diverse endemic herbivore radiations, the leafhoppers (Hemiptera: Cicadellidae: *Nesophrosyne*), as a model system to understand the role of host-plant use in insect diversification. A comprehensive phylogeny is reconstructed to examine the origins, species diversification, and host-plant use of the native Hawaiian leafhoppers. Results support a monophyletic *Nesophrosyne*, originating from the Western Pacific basin, with a sister-group relationship to the genus *Orosius*. *Nesophrosyne* is characterized by high levels of endemicity according to individual islands, volcanoes, and geologic features. Clades demonstrate extensive morphologically cryptic diversity among allopatric species, utilizing widespread host-plant lineages. *Nesophrosyne* species are host-plant specific, demonstrating four dominant patterns of specialization that shape species diversification: 1) diversification through host switching; 2) specialization on widespread hosts with allopatric speciation; 3) repeated, independent shifts to the same hosts; and, 4) absence or low abundance on some host. Finally, evidence suggests competing herbivore radiations limit ecological opportunity for diversifying insect herbivores. Results provide evolutionary insights into the mechanisms that drive and shape this biodiversity.

Key Words: Hawaii; Cicadellidae; *Nesophrosyne*; Origin; Cryptic Species; Host-Plants; Phylogeny

Introduction

The relationship between plants and insects is one of the greatest evolutionary stories in the history of life on earth. Their importance in global terrestrial ecosystem functioning is self evident, as both represent the most abundant life on the planet (Schoonhoven et al. 2005). At least half of all known insects feed on plants; however, this number is an underestimate, as up to 90% of insect life has yet to be described (Strong et al. 1984; Grimaldi and Engel 2005). While plant-insect interactions have received much attention and are easily manipulated in experimental studies, there have been few broad-scale phylogenetic studies for circumscribed herbivorous groups and their ecological associations (Schoonhoven et al. 2005; Janson et al. 2008). As a result, the evolutionary role of interspecific interactions in promoting herbivorous insect and plant diversification, at both the global and local scales, remains unclear (Schoonhoven et al. 2005; Janson et al. 2008; Roderick and Percy 2008).

In order to assess the evolutionary importance of plant-insect interactions in the evolution of both groups, well-developed phylogenetic hypotheses and circumscribed host-plant information are necessary for specific taxonomic groups. These data can be difficult to acquire in broadly distributed, diffuse systems with continental distributions. Oceanic islands offer unique, tractable natural laboratories to circumscribe and study plant-insect evolution, due to their discrete, isolated, and replicated nature (MacArthur and Wilson 1967; Grant 1998). The Hawaiian archipelago is unrivaled in this regard, with its biota derived almost entirely *in situ*, resulting in unparalleled rates of endemism: 99% for arthropods and 89% for plants (Wagner and Funk 1995; Miller and Eldridge 1996; Wagner et al. 1999). These factors provide the

opportunity, especially with plant-insect interactions, to develop an understanding of the forces that drive and shape biodiversity. Furthermore, the insular nature of island radiations offer the ability to biologically and evolutionarily contextualize archipelagic species diversification by locating the source of origin for endemic lineages (Carlquist 1966). Expanding this knowledge for island groups yields a model for understanding how regional biodiversity is evolutionarily assembled and maintained.

Previous studies on herbivorous insects have generally focused on several iconic model systems, including Lepidoptera (Ehrlich and Raven 1964; Pellmyr 2003), Coleoptera (Farrell and Mitter 2008; Farrell and Sequeira 2004), Psyllidae (Percy et al. 2004), *Rhagoletis* fruit flies (Berlocher 2000), and fig wasps (Silvieus et al. 2008). While the developed knowledge of these systems provides invaluable insights and a comparative framework, results may not be generally applicable to other groups (Janson et al. 2008). Remarkably, one of the largest gaps in our understanding includes the hemipteroid lineages in Auchenorrhyncha, which contain some of the largest obligate host-plant restricted groups known (Grimaldi and Engel 2005). In particular, the leafhoppers (Cicadellidae) provide a unique, and heretofore overlooked, system with which to examine host-insect evolution. Cicadellids represent one of the largest insect families and compose a dominant element of terrestrial ecosystems globally (Oman et al. 1990; Zahniser and Dietrich 2008). Leafhoppers have been considered excellent models to understand ecological and biogeographic mechanisms of species diversification due to their high levels of host-plant specificity, limited dispersal, and high rates of local endemism (Hamilton 1997; Nielson and Knight 2000; Felix and Mejdalani 2011). Yet despite their ecological dominance, and highly adapted phytophagous lifestyle, species-level diversification patterns and ecological associations remain largely unknown, particularly in the largest subfamily, Deltocephalinae (Zahniser and Dietrich 2010).

The presented research focuses on the endemic Hawaiian leafhopper genus *Nesophrosyne* (Deltocephalinae: Fig. 1). *Nesophrosyne* represents one of the most diverse and ecologically dominant herbivore radiations on Hawaii. The genus contains 72 described species; however, its total diversity remains mostly undescribed (Zimmerman 1948; Bennett and O'Grady 2011). Species are distributed across all high islands, filling niches in coastal to subalpine habitats. *Nesophrosyne* are obligate phloem feeders, ovipositing and developing on particular host-plants, and are highly host specific, utilizing 75% of the most ecologically dominant and species-rich endemic genera (Kirkaldy 1907, 1910; Osborn 1935; Zimmerman 1948; Bennett and O'Grady 2011; Wagner et al. 1999). They further demonstrate dramatic morphological adaptations both to local climate and to host-plant morphology (Zimmerman 1948). The endemic nature and host specificity of *Nesophrosyne* offers a discrete and diverse system to examine the role of host-plant associations in insect diversification.

This study investigates the origin, species diversity, and host-plant use of *Nesophrosyne* in a molecular phylogenetic context. The principal question guiding this work is the roll of host-plant specialization in shaping ecological and species diversification in phytophagous insects. This study is one of the first aimed at understanding genus-wide host-plant associations in a molecular systematic framework within Cicadellidae and for native Hawaiian hemipteroid radiations. Results establish a general model for contextualizing species diversification and the role of host-plant associations in the radiations of herbivorous insects.

Methods

Material collected

Ingroup taxon sampling and species delimitation for phylogenetic analyses was conducted under the following criteria (in the following order): i) morphological diagnosis of described species and identified undescribed species, ii) ecological species diagnosis based on host-plant associations, and iii) geographical diagnosis of species distributed across the biogeographic provinces of the Hawaiian Islands. These criteria were used to delimit initial species hypothesis, which were then objectively evaluated with the Cytochrome Oxidase II (COII) locus. The use of COII for species delimitation in *Nesophrosyne* was tested in Bennett and O'Grady (2011) for the *kanawao* species group (Clade M in this study; see Results, Fig. 3). They found that COII reliably reconstructs highly supported, reciprocally monophyletic clades for both morphologically diagnosable species and cryptic sibling species with large disjunct ranges (*e.g.,* the volcanoes of Maui and Hawai`i Island). Intra-specific species variation was found to fall within 2-3% divergence across large species' ranges (*e.g., Nesophrosyne aakokohaikea* endemic to Mauna Loa). Thus, individuals in this study with identical COII haplotypes or < 2-3% difference, were removed from further molecular analyses. Final multi-gene, phylogenetic results were used as a fourth objective criterion to evaluate species hypotheses (see Results). In total, 363 individuals were screened, in order to delineate species boundaries and to test host-plant associations (data not shown). A total of 191 *Nesophrosyne* species among 198 samples were identified in this study, representing 48 of the 72 currently described species, and 143 potentially new species (SI Table 1).

Material included in molecular analyses was field collected by sweep-netting individual plants and placed directly into 95% ethanol and labeled with a four-digit field barcode (*e.g.,* GB-001.7). Species identifications followed Zimmerman (1948) and original descriptions (Kirkaldy 1907, 1910; Osborn 1935; Bennett and O'Grady 2011). It is important to note that *Nesophrosyne* has not heretofore received systematic treatment, or a focused taxonomic effort, and reliable identifications for much of the described diversity are difficult and often impossible (Zimmerman 1948; Bennett and O'Grady 2011). Furthermore, species described by Kirkaldy (1907, 1910) and Osborn (1935) are based on inconsistent premises (*e.g.,* single specimens, males, females, or teneral specimens) and further consist of short, often uninformative descriptions (see Kirkaldy 1910). Species unable to be positively identified are considered to be new, and are designated with an appended DNA barcode (*e.g.,* "*N. sp.123"*). Material for DNA extraction followed a non-destructive vouchering protocol in order to preserve specimens for designation as type material. Morphotype vouchers have been designated for each species from the material used in phylogenetic analyses, and include the dorsal habitus and genitalia.

In order to determine the geographic and taxonomic origin for *Nesophrosyne*, taxon sampling included a total of 12 genera (16 individuals) in Deltocephalinae subfamily from around the Pacific Basin (SI Table 1). Taxon selection was informed by a) previous systematic work on the Deltocephalinae subfamily, targeting members of the Opsiini tribe and related genera (Zahniser and Dietrich 2010); and, b) proposed sister genera to *Nesophrosyne*, including *Scaphoideus* (Osborn 1935) and *Osbornellus* (Zimmerman 1948). The strongest candidate for the closest relative of *Nesophrosyne* is the genus *Orosius*, which shares membership in the Opsiina subtribe (Dai et al. 2010; reviewed in Bennett and O'Grady 2011). *Orosius* has Western Pacific Rim distribution (*e.g.* Oceania, South East Asia, and Africa), and was sampled from Australia for

this study (SI Table 1). Species in the genus *Deltocephalus* (Tribe: Deltocephalini) were specified as phylogenetic outgroups.

Host-plant associations

The host-plant associations of *Nesophrosyne* were resolved in the field from collections conducted by the authors. Field collected data were combined with historical collections housed in the Bernice P. Bishop Museum, Honolulu, HI; the University of Hawai`i, Manoa Insect Museum; original literature describing species and paratype collections, including material in the British Museum of Natural History, London (Kirkaldy 1907, 1910; Osborn 1935); and, a large collection (~1500 specimens) provided by D. Polhemus from the Smithsonian Institution's National Museum of Natural History, Washington, D.C. The dominant host-plant of a leafhopper species was inferred under the following criteria: a) a positive host identification to the plant genus level; b) confirmation of host associations through repeated collection of a leafhopper species from the same plant (*i.e.,* singletons are generally treated as unknown); and c) phylogenetic relationships support a proposed host, unless contradicted by field data. Some species may be capable of utilizing secondary hosts and were thus considered; however, there is considerable evidence that most if not all species are host-plant specific (Kirkaldy 1907, 1910; Osborn 1935; Zimmerman 1948; Bennett and O'Grady 2011), which is a common pattern among other herbivorous insects on Hawaii (Asche 1997; Roderick and Percy 2008; Polhemus 2011), and a general expectation for herbivorous insects world-wide (Schoonhoven et al. 2005).

Host-plants were field identified to family and genus according to current taxonomic standards provided in the Manual of the Flowering Plants of Hawai`i (Wagner et al. 1999). Plants that could not be field determined were photographed and later identified. Species-level identifications were made when possible; however, this requires plants to be in flower or fruit, which was not always available. Furthermore, the species-level taxonomy for some Hawaiian plant lineages is not well worked out (Wagner et al. 1999).

Sequencing and alignment

Total genomic DNA was extracted from a cross section of the anterior abdominal segments, using Qiagen DNeasy extraction kit (Qiagen Corps). A total of six genes were amplified from mitochondrial (*Cytochrome Oxidase I & II,* and 16S), nuclear (*Histone 3* and *Wingless*), and obligate betaproteobacterial endosymbiont (16S: CoBE-16S) genomes. Bacterial endosymbiont genomic data were selected because they have been increasingly incorporated in inference of non-model organism evolution since obligate symbionts are transovarially transmitted and show ancient co-cladogenesis (Lozier et al. 2007; Andersen et al. 2010; Moran et al. 2008). Specifically, bacterial endosymbionts have been demonstrated to show strict co-phylogenetic correspondence in other leafhopper-bacteria systems (Takiya et al. 2006). The obligately associated betaproteobacteria in *Nesophrosyne* is related to other betaproteo lineages found throughout Auchenorrhyncha, including '*Candidatus* Zinderia insecticola' from spittlebugs also in the Cicadomorpha infroadorder (Bennett unpub. data; McCutcheon and Moran 2010), as well as in multiple genera from across the Deltocephalinae subfamily (presented here and see Noda et al. 2012), further supporting the ancient co-cladogeneic association for these lineages. Polymerase Chain Reaction conditions and primers were adapted from previous studies (see Table 1) and optimized for *Nesophrosyne*. Reactions began with an initial 5 min

denaturation step at 95°C; followed by a 35-cycle regime of 95°C for 30 sec, 54-64°C for 1 min (annealing optimized for each gene: Table 1), and 72°C for 1min (extension phase); and, a final 72°C extension for 5 min. For COI, a stepwise PCR reaction was used with a three-stage cycle regime as follows: 95°C for 30s, 60°C for 1min, and 72°C for 1 min, for 10 cycles; 95°C for 30s, 56°C for 1min, and 72°C for 1 min, for 10 cycles; and 95°C for 30s, 54°C for 1min, and 72°C for 1 min, for 5 cycles. Primer pairs that did not work for some taxa were redesigned and optimized using Primer3 (Table 1; Rozen et al. 2000). Successful PCR amplifications were cleaned using the ExoSAP-IT protocol (USB). Both forward and reverse directions were sequenced at the U.C. Berkeley, Barker Sequencing Center, using an ABI 3730 capillary sequencer. Sequences were submitted to GenBank for public archiving (SI Table 1).

Sequenced contigs were aligned and edited in Geneious v5.1 (Drummond et al. 2010) and identity checked on GenBank. Different alignment strategies were employed based on the genomic locus of each gene. All protein-coding genes were aligned using MUSCLE v3.5 (Edgar 2004), conceptually translated into amino acid sequences, and checked for frame-shift mutations and internal stop codons. 16S mtDNA contains highly variable loop regions and was aligned using Probalign v1.2, which has demonstrated superior performance over other available alignment methods (Roshan and Livesay 2006). The CoBE-16S locus was aligned using the RDP 10, which informs alignment strategy with secondary ribosomal folding structure (Cole et al. 2008). Sequence alignments of both 16S and CoBE-16s revealed highly variable loop regions containing large insertion-deletions for which homologous statements were not trivial, and were removed as follows: 16S base pair regions 30-38, 165-195, 290-305, 325-361, and 590-604; and, CoBE-16S regions 72-78 and 778-792. Preliminary phylogenetic analyses further indicated that the inclusion of the loop regions reduced support at more basal node, indicating conflict between these regions and the larger alignment (data not show). Aligned gene matrices were concatenated into a full data matrix, pending assessment of phylogenetic congruence (see below).

Phylogenetic analyses

Phylogenetic analyses were performed on a data matrix consisting of 214 taxa and 6 loci, comprising 3520 characters. Species relationships were inferred using maximum likelihood (ML) and Bayesian methods. All loci were initially examined individually and in combination by genome to determine their suitability for concatenation into a single data matrix. In particular we evaluated the influence of the CoBE-16S on the inference of phylogeny, since it offers a novel locus for cicadellid systematics. Supplementary Information Figures 1 and 2, show the phylogenetic results for the alignment excluding the CoBE-16S locus and for the CoBE-16S gene tree, respectively. Both recover the same general clade and species level relationships found in the complete data alignment, although basal and mid-level support is greatly reduced relative to the total dataset. The CoBE-16S locus is a relatively conserved gene, increasing mid-level and basal nodal support (*e.g.,* see Results Fig. 3 and SI Fig. 1, Clades A, M, K, and I, among others). The effect of missing data was also assessed by reconstructing phylogenetic relationships with data matrices that progressively removed loci with the highest percent missing data (see SI Table 1 for gene sampling). All data assessments and verifications were performed in RAxML v7.2.7 (Stamatakis et al. 2008), described below (results not shown). In general, species-level relationships are resolved for fast evolving mtDNA loci (COI and COII), but deeper relationships are unresolved. Conversely, nuDNA and ribosomal genes show mixed resolution at deeper nodes and are unable to resolve species-level relationships. Removal of loci with missing data did not

change species or clade level relationships, but basal support progressively declined. Thus, all loci were concatenated into a single data matrix for full phylogenetic analyses.

Gene partitioning has become a required consideration for properly analyzing large multi-gene datasets and directly optimizes information contained within large complex data matrices, which is particularly useful for rapid radiations such as those on islands (Brandley et al. 2005; Li et al. 2008). The optimal partitioning strategy for this study was determined in a ML framework, using RAxML v7.2.7 on Cipres (Stamatakis et al. 2008; Miller et al. 2009). RAxML was chosen due to the computational complexity of the dataset and available computing power (Stamatakis 2008). Molecular data were increasingly partitioned, according to the biological elements from a single partition (concatenated) to fourteen partitions (codons, 16S, and CoBE-16S: Table 2). RAxML was run under the GTR-GAMMA model for 1000 bootstrap iterations and final ML search. For each ML reconstruction, log likelihood scores were used to evaluate partition performance with the Akaike Information Criterion (AIC) and AIC weights (Table 2: Burnham and Anderson 2002). The AIC overwhelmingly selected a fully partitioned analysis, which contributes to a growing body of literature that suggests codon and gene identity are important considerations in phylogenetic analyses of multi-gene datasets (Brandley et al. 2005).

Bayesian phylogenetic inference was completed using MPI-MrBayes v3.1.2 on the Abe TeraGrid in Cipres (Ronquist and Huelsenbeck 2003; Miller et al. 2009). Likelihood models of base-pair evolution were determined for each partition element with the Bayesian Information Criterion in MODELTEST v3.7 (Posada and Crandall 1998). Since MrBayes is unable to incorporate some models of evolution, the closest model to the one selected with Modeltest that could be implemented, with the necessary parameters (Table 3), was used for each Bayesian phylogenetic analysis. All data partitioned elements were unlinked, assigned the appropriate model of molecular evolution, and branch lengths set to vary proportionally across partitions. Based on preliminary analyses, chain temperature was adjusted down (t=0.1) to facilitate convergence on the posterior optima. Two independent searches of the posterior distribution were run with four chains each for a total of 17.322×10^6 generations, sampled every 1000^{th} iteration. Runs were monitored throughout their length, using the average standard deviation of the split frequencies (ASDSF) and the cumulative function in AWTY (Nylander et al. 2008). The run was terminated after achieving an ASDSF of 0.03 (an ASDSF = 0.05 was used as an approximation of convergence of large datasets; Ronquist et al. 2011). Final convergence and burn-in were determined by plotting outputs in Tracer v1.5 and AWTY, and assessing the potential scale reduction factor (Nylander et al. 2008; Rambaut and Drummond 2009). A 50% majority rule consensus tree was constructed from post burn-in iterations.

Results

Host-plant associations

A total of 4118 specimens with positive host-plant information were examined. The dominant host-plant associations were determined for 175 *Nesophrosyne* species, which utilize 33 plant genera in 21 families (SI Table 1). Species are highly host-plant specific. The four most dominant host-plant associations broken down by plant family and genera are the following: Rubiaceae (*Psychotria*, *Coprosma*, *Bobea*, *Hedyotis*), Urticaceae (*Pipturus*, *Urera*, *Boehmeria*, and *Touchardia*), Hydrangeaceae (*Broussasia*), and Sapindaceae (*Dodonaea*). There is evidence that a few *Nesophrosyne* species may use more than one host, including the following: *N.*

furculata on *Lobelia* sp. (Campanulaceae) and *Coprosma* sp.; *"N. sp.26"* and *N. umbratilis* on *Hedyotis* sp. and *Psychotria* sp.; and, *N. pele* on *Melicope hawaiiensis* (Rutaceae) and *M. radiata*. Results also indicate that *"N. sp.132"* is associated with the fern genus *Sadleria* (Blechnaceae), which is a unique association for the genus. The dominant host-plant associations were mapped across the tips of phylogenetic results in order to determine the relationships of *Nesophrosyne* species and their associations with particular hosts

Phylogenetic results

Phylogenetic results from ML and Bayesian trees were combined using Archaeopteryx v0.957 beta, which provides a visual tool for comparison of tree topologies (Han and Zmasek 2009). Both methods yielded similar topologies, providing high support for a monophyletic *Nesophrosyne* and the relationships among other genera (Fig. 2). Basal nodes are generally well supported (BS=68-81, PP=1); however, there is a large unresolved polytomy at mid-depth in the tree. Differences between trees were confined to branches that received low or no support with both methods, except in one instance where ML supported a bifurcation between taxa "*N. sp.126"* and *N. giffardi* + *N. giffardi interrupta* that was not supported by the Bayesian analysis. Bayesian results provide higher support for clades at deeper nodes, and are selected as the reference tree for inference of relationships between *Nesophrosyne* species (Figs. 2-5).

Origin of Nesophrosyne *and higher taxonomic relationships*

Figure 2 shows the relationships of *Nesophrosyne* and Deltocephalinae genera. Both ML and Bayesian analyses provided strong support for the sister relationship of *Nesophrosyne* to the genus *Orosius* from Australia. The Opsiini genus *Lampridius* from Thailand, is placed sister to the *Nesophrosyne* + *Orosius* clade. The monophyly of Opsiini is not supported by the inclusion of a large clade of Deltocephalinae genera currently assigned to the Athsyanini tribe (*Scaphoidella*) or of unplaced tribal status (*Mimotettix, Scaphoideus, Phlagotettix, Osbornellus*), which is additionally supported by the convergent and variable nature of the proposed synapomorphic bifurcate aedeagus at higher taxonomic levels (Dai et al. 2010).

3.4 Species relationships and host-plant use

Nesophrosyne species-level relationships are well supported and assignable to clades, providing adequate resolution to assess host-plant associations, species diversity, and areas of endemicity (Fig. 3). Aside from Bennett and O'Grady (2011), there has been no previous systematic or taxonomic effort treating the relationships within *Nesophrosyne*, and no precedence exists for comparison of species relationships. *Nesophrosyne* forms large multi-island clades according to host-plant associations and morphological types, which will be described as species groups and subgroups in an upcoming revision. In order to simplify discussion of results, well-supported clades according to the Bayesian analyses are assigned alphanumeric names (Clade A-N; Fig. 3). Broadly, island species distributions are complex, with species occurring on older islands (Kaua`i or O`ahu), representing basally diverging lineages for some clades (A, C, F, H, K, L, M), but more nested lineages in others (D, G, J, N). Host-plant specialization can be summarized as clades characterized by multiple host transitions across plant genera and families (A, C, G, J, L, O); or, clades associated with single widespread host-plant lineages (A, Urticaceae: *Pipturus & Urera*; E, Sapindaceae: *Dodonaea*; I, F, C; Rubiaceae:

Coprosma and *Psychotria*; K, Rutaceae: *Melicope*; M, Hydrangeaceae: *Broussaisia*; H, Myrtaceae: *Metrosideros*; O, Myrsinaceae: *Myrsine*; and, D, Scrophulariaceae: *Myoporum*).

Nesophrosyne is basally split into two parallel radiations (Fig. 3): Clade A (Figs. 3 & 4: BS=73, PP=.94) and its sister group Clade B (BS=68, PP=.99). Clade A comprises all species associated with genera in the plant family Urticaceae (*Urera, Touchardia, Boehmeria*, and *Pipturus*) throughout all high islands, and a single well-supported transition by *N. heopoko* to *Broussaisia arguta* (Hydrangeaceae) on Kaua`i (Figs. 3 & 4: BS=99, PP=1). Species-level relationships are well supported, with the exception of species associated with *Urera*. Species specializing on *Pipturus* are paraphyletic, including a clade comprising three monophyletic groups restricted to O`ahu, Maui Nui, and Hawai`i Island (BS=100, PP=1), and a clade comprising "*N. sp.17"* and *"N. sp.120"* on Kaua`i that is sister to species associated with *Boehmeria* and *Broussaisia*.

Clade B represents a large radiation associated with all other plant groups (Fig. 3). The basally diverging Kaua`i lineages of that radiation are associated with several host-plant genera and families (*e.g.,* Rubiaceae: *Psychotria*, Goodeniaceae: *Scaevola*, and Campanulaceae: *Clermontia*), most with high support (BS=41-82, PP=.63-1). The remainder of Clade B comprises all other lineages, which form clades emerging from a large mid-depth polytomy. The remaining clades are described below, to illustrate species relationships and unique patterns among *Nesophrosyne* lineages and clades.

Clade C (Fig. 3): Clade C (BS=35, PP=1) comprises a large group of small-bodied leafhoppers spread throughout the islands. Support for species relationships are mixed, with most receiving high support (BS=32-100, PP= .64-1). Host-plant transitions in the group are common, and species are associated with 15 plant genera in 11 families (Fig. 3; SI Table 1). The basally diverging lineages are endemic to Kaua`i, and are associated with plant genera *Tetraplasandra* and *Cheirodendon* (Araliaceae: BS=100, PP=1). The remaining species have diverse host associations and are distributed across all islands, which includes: A multi-island clade (*e.g., N. comma* and *N. oceanides*) associated with sister species, *Psychotria mariana* and *P. hawaiiensis* (Rubiaceae: BS=81-100, PP=1); a Kaua`i clade restricted to *Psychotria* and *Hedyotis* (Rubiaceae), including the multi-host associated *"N. sp.26"* (BS=100, PP=1); a Kaua`i and O`ahu clade, with basally diverging Kaua`i species associated with *Syzigium* (Myrtaceae), and O`ahu species associated with Rubiaceae genera (*Bobea*, *Coprosma*, and *Psychotria*: BS=49-64, PP=.79-.96); Clade D, which is found on all islands associated with *Myoporum sandwicense* (Scrophulariaceae: BS=61, PP=1), comprising morphologically cryptic species; a diverse clade of Hawai`i Island species associated with 5 families (*e.g. N. ignigena* on Aquifoliaceae: *Ilex, "N. sp.268"* on Araliaceae: *Cheirodendron*, and *N. mabae* on Ebenaceae: *Diospyros*, etc.: BS=91-100, PP=.82-1); a Maui Nui (Moloka`i, Lana`i, and Maui) group associated with *Scaevola* (Goodeniaceae,) and *Wikstroemia* (Thymelaeaceae: BS=94-100, PP=.95-1); and, finally, an O`ahu associated clade with its basally diverging lineage associated with *Wikstroemia* and other with *Psychotria* (BS=95-100, PP=1).

Clade E (Figs. 3 & 5): All species in Clade E are restricted to the widespread host-plant *Dodonaea viscosa* (Sapindaceae). Taxa associated with *D. viscosa* do not constitute a monophyletic group, with an independent transition of *"N. sp.29"* on Kaua`i (Fig. 3, Clade C: BS=91, PP=1). Lineages are divided into two parallel groups, including one on Maui, Moloka`i, and O`ahu (BS=56, PP=.8), and the other on Maui and Hawai`i Island (BS=68, PP=1); Maui species are basally divergent in both cases. Lineages occurring on Moloka`i and O`ahu are closely related (BS=100, PP=1), with *N. maritima* forming a low elevation, multi-volcano

endemic (BS=71, PP=.95). Gross morphology is similar in all species, but forms two morphologically cryptic clades, one light and the other dark, corresponding to Maui-Oahu group and Maui-Hawaii Island group, respectively.

Clade F (Fig. 3): Clade F (BS=82, PP=1) comprises several species in the former *Nesophrosyne* (*Nesoreias*) subgenus. All species are associated with the plant genus *Psychotria* (Rubiaceae). The basally diverging species, *"N. sp215"*, is endemic to O`ahu, while the rest are endemic to Hawai`i Island (*e.g., N. insularis*, *N. eburneola*, and *N. marginalis*: BS=98, PP=1).

Clade G (Fig 3): Clade G, aside from two species with unknown host associations, is restricted to plant genera in the plant family Rubiaceae. The genus level host associations are predominantly split between *Coprosma* and *Hedyotis*. Species are spread throughout all high islands, with basally diverging lineages found on Maui and restricted to *Coprosma* (BS=100, PP=1). The Kaua`i species, *N. umbratilis*, is evidently associated with *Psychotria* and *Hedyotis*, and placed in a clade with Moloka`i and Maui species with unknown host associations (BS=43, PP=.70). The remainder of Clade G forms highly supported, reciprocally monophyletic groups comprising parallel radiations associated with *Hedyotis* (BS=97, PP=1) and *Coprosma* (BS=100, PP=1). The *Hedyotis* associated clade has a Maui Nui and Hawai`i Island distribution, while the *Coprosma* associated clade is restricted to Hawai`i Island. Species in the *Coprosma* associated clade (*e.g., N. pluvialis*, *"N. sp.278",* and *"N. sp. 280"*) form morphologically cryptic species restricted to single volcanoes and volcanic features (*e.g.,* volcanic flanks).

Clade H (Fig 3): Clade H comprises the only species associated with *Metrosideros polymorpha* (Myrtaceae: BS=56, PP=1). Species are spread from O`ahu through Hawai`i Island. O`ahu species demonstrate endemicity according to volcanic ranges (BS=93, PP=1). Species further demonstrate dramatic external morphological evolution: *N. notatula* is a uniquely brown speckled species, while *"N. sp.36"* and *N. sp.345"* are reddish in color without extensive color patterning.

Clade I (Figs. 3 & 5): Clade I comprises species associated with plant genus *Coprosma* (Rubiaceae: BS=69, PP=.99), occurring on Moloka`i, Maui, and Hawai`i Island. Species relationships are all highly supported (BS=80-100, PP=.99-1). Maui species are rendered paraphyletic by *"N. sp.225"* on Moloka`i and the Hawai`i Island endemics. Hawai`i Island species constitute a monophyletic group (BS=92, PP=1) and are single-volcano endemics. Species in Clade I are similar morphologically, and indistinguishable in some instances (*e.g., "N. sp.302", "N. sp.306",* and *"N. sp.277*). *Nesophrosyne haleakala*, restricted to the subalpine Haleakala crater floor on Maui, is the only brachyapterous species with a thickened cuticle.

Clade J (Fig. 3): Species in Clade J are spread from O`ahu through Hawai`i Island (BS=48, PP=91). Species demonstrate extensive host transitions among 10 plant genera in 7 families (*e.g.,* Rubiaceae: *Coprosma & Hedyotis*, Campanulaceae: *Lobelia* & *Clermontia*, Hydrangeaceae: *Broussaisia*, Gesneriaceae: *Cyrtandra*, *etc*.). Branch support is mixed, with many branches receiving low support, resulting in a polytomy that contains species endemic to O`ahu, Moloka`i, and Maui (*e.g., N. monticola*, *"N. sp.189"*, and *"N. sp.137"*). Several clades receive moderate to high support, which include: A *Coprosma* associated group, endemic to Moloka`i and Maui, including the previously described *N. anguilifera* and *N. obliqua*, which share unique external markings, but differ dramatically in head morphology (BS=100, PP=1); a low-moderately supported Maui Nui clade with diverse host associations including *N. furculata*, which has a multi-host association with *Coprosma* and *Lobelia* (BS=24, PP=.92); a moderately supported O`ahu endemic clade (BS=58, PP=.93) with multiple host associations (*e.g., N. oreadis* on the host-plant Thymelaeaceae: *Wikstroemia*, and *N. monticola* on *Coprosma*); and,

finally, a highly supported Maui Nui Clade (BS=100, PP=1), with extremely unique host associations, including the single association of "*N. sp.132"* with *Sadleria* fern (Blechenaceae), *"N. sp358"* with *Lobelia*, and *"N. sp.137"* with *Cyrtandra*, each with unique adaptive morphology (Zimmerman 1948)

Clade K (Figs. 3 & 5): Species in Clade K extend throughout the Hawaiian archipelago, except Kaua`i, and are associated with the genus *Melicope* (Rutaceae: BS=28, PP=.99). Species level relationships are highly supported (BS=68-100, PP=.98-100 ). O`ahu endemic *N. palolo* forms the most basally diverging lineage for the group (BS= 28, PP=.99). Species on O`ahu and Maui do not constitute single clades by island (BS=70-92, PP=.98-1), while species occurring on Hawai`i Island are a monophyletic group and have radiated among volcanic peaks (BS=100, PP=1). Members of this group have transitioned among different *Melicope* species in this range. External morphological coloration varies extensively between islands, but is cryptic within islands.

Clade L (Fig. 3): Species in Clade L are endemic to the older Islands, Kaua`i and O`ahu (BS=3, PP=.99). Clade and species support is high, with host-plant associations mixed and partly unknown. The O`ahu species are placed sister to each other (BS=100, PP=1), and both associated with *Nestegis* (Oleaceae). Species endemic to Kaua`i form a highly supported clade (BS=100, PP=1), and the two known host associations include *Psychotria* (Rubiaceae) and *Wikstroemia* (Thymelaeaceae).

Clade M (Fig. 3 & 5): Clade M, the *kanawao* species group, is the only host-associated clade that has been described in *Nesophrosyne* (Bennett and O'Grady 2011). Species in this group are restricted to the multi-island endemic plant species *Broussaisia arguta* (Hydrangeaceae) across all high islands (BS=68, PP=.99), and are the only taxa of *Nesophrosyne* known from *B. arguta* except *N. heopoko* on Kaua`i (Figs. 3 & 4, Clade A) and *N. magnaccai* on Moloka`i (Fig. 3, Clade J), which represent independent host transitions. All species constitute single-volcano endemics (BS=76-100, PP=.94-1). Species form monophyletic, morphologically cryptic groups according to island (Bennett and O'Grady 2011).

Clade N and O (Figs. 3 & 4): Species in Clade N form a multi-island radiation, excluding Kaua`i, in which host-plant transitions are common (BS=61, PP=1). Internal species-level support is mixed (BS=13-100, PP=.66-1), with most relationships receiving high support. Clade N comprises three groups with unique associations across multiple distantly related host-plant genera, including a paraphyletic multi-island association with *Psychotria* (Rubiaceae: BS=28-77, PP=.85-1), a Maui restricted clade with mostly unknown host associations (a single association with Dipentodontaceae: *Perrottetia*: BS=100, PP=1), and a Hawai`i Island restricted radiation on *Clermontia* (Campanulaceae: BS=100, PP=1). Clade O represents a monophyletic association of species associated strictly with the plant genus *Myrsine* (Myrsinaceae: BS=100, PP=1). Species in each group form single-volcano endemics. A highly supported fifth group, restricted to O`ahu, has specialized on at least four different plant genera: *Melicope* (Rutaceae), *Scaevola* (Goodeniaceae), *Hedyotis* (Rubiaceae), and *Cloaxylon* (Euphorbiaceae), all with high support (BS=93-100, PP=.94-100).

Discussion

Nesophrosyne *origin and higher taxonomic relationships*

Determining the origin for endemic Hawaiian radiations is difficult due to the geographic and temporal scale of the problem, which encompasses at least the entire Pacific Basin and millions of years. As a result, the origins for several of the largest endemic Hawaiian insect radiations remain unknown (*e.g., Drosophila* and *Hyposmocoma* moths: Rubinoff 2008; O'Grady and DeSalle 2008). Phylogenetic results presented here, support the hypothesized sister relationship and shared origin between *Orosius* and *Nesophrosyne* (Fig. 2: Ghauri 1966; Linnavuori 1975). The genitalic characters for species in each genus are morphologically similar, which led to a temporary synonymy of the two (reviewed in Bennett and O'Grady 2011). Previous Hawaiian workers suggested other potential sister groups to *Nesophrosyne*, including *Osbornellus*, *Scaphoideus*, and *Nephotettix* (Kirkaldy 1910; Osborn 1935; Zimmerman 1948), which do not share internal genitalic characters with *Nesophrosyne* and are placed in distantly related tribes or unnamed groups (Ghauri 1971; Barnett 1976; Dominguez and Godoy 2010; Zahniser and Dietrich 2010).

The inferred shared ancestry of *Nesophrosyne* and *Orosius* provides the potential to biologically and evolutionarily contextualize species diversification on Hawai`i. *Orosius* is widely distributed throughout Pacific-Oceania region, South East Asia, and Africa (Ghauri 1966). Many species are highly dispersive, with geographically wide distributions (*e.g., O. argentatus*: Oceania through Africa), and are polyphagous economic pests, vectoring a number of plant diseases (Larsen and Walter 2007). Plant associations are largely unknown for *Orosius* species; however, the economically important species, *O. argentatus* and *O. orientalis*, feed broadly across many plant families (Ghauri 1966; Grylls 1979; Larsen and Walter 2007). These traits, shared among of some *Orosius* species (Ghauri 1966), increase the likelihood of long-distance dispersal to remote archipelagos and post-colonization success. Thus, evidence suggests *Nesophrosyne* may have originated from a widespread, polyphagous ancestor.

An inferred Western Pacific origin for *Nesophrosyne*, based in part on the natural distribution of *Orosius*, is further supported by the placement of the genus *Lampridius* (Tribe: Opsiini) from Thailand, sister to the *Orosius*-*Nesophrosyne* clade (Fig. 2). Arrival to Hawaii from the West suggests two potential routes for long-distance dispersal: aerial dispersal along the East-West Sub Tropical Jet Stream (STJS), or oceanic dispersal through flotation. Wind-dispersal along the STJS is predicted for taxa that can be easily swept into and carried by high-altitude winds (Geiger et al. 2007; Gilliespie et al. 2011). While cicadellid species demonstrate low dispersal rates (Nielson and Knight 2000), they are capable of flight, easily swept up in major wind currents, and some are known to be migratory (Nault and Rodriguez 1985). Gillespie et al. (2011) argue that aerial dispersal is unlikely in animal taxa due to harsh conditions, and lineages with a western origin alternatively relied on dispersal via oceanic currents. This route is available to insects like members of *Nesophrosyne*, which deposit their eggs directly into plant leaves or otherwise spend part of their life stages in vegetation. This medium can provide a buffer against harsh seawater, especially if the leaves are part of a larger mass of vegetative material. However, this mode of dispersal requires a potentially prohibitive 2-3 week journey by sea, whereas aerial dispersal along the STJS can be accomplished in 2-4 days (Geiger et al. 2007; Gillespie et al. 2011).

Nesophrosyne *diversity and endemicity*

Nesophrosyne is a monophyletic radiation of at least 190 species identified in this study, which represents the most comprehensive assessment to date. Given that some described species were not collected here, the extant species diversity is likely to be well over 200. Historically, 72 species have been described, but unfortunately much of the original taxonomic work is flawed, which has confused previous understanding of *Nesophrosyne* diversity and classification (Zimmerman 1948; Bennett and O'Grady 2011). Results from this study illuminate previously unknown species diversity, providing clear ecological and evolutionary background for species delimitation and genus-wide revision. *Nesophrosyne* is the only endemic leafhopper group to have radiated into hundreds of species.

Among the herbivorous hemipteroid groups endemic to Hawaii, *Nesophrosyne* is perhaps the most diverse, exceeding endemic Fulgomorpha planthoppers (Roderick and Percy 2008; Hoch 2006; Asche 1997) and the largest plantbug genus, *Orthotylus* (Miridae: Polhemus 2011). Levels of endemism are exceptionally high, with species forming single-island endemics, with the exception of only a few that are more widespread. Lineages further demonstrate high levels of local endemicity, according to geologic sub-partitioning of islands vis-à-vis volcanic peaks and shield flanks, landslides, and eroded valleys. This result provides insights into the unique biogeographic provinces of the Hawaiian Islands. For example, Haleakala Crater (Maui) has a diversity of locally endemic species, including *N. kaupoi*, *N. haleakala*, and *"N. sp.48"* among others, each specializing on unique host-plants (Fig. 3 and SI Tables 1 & 2). The high degree of local endemism exhibited by *Nesophrosyne* is found in other taxonomic groups of arthropods (*e.g., Hyposmocoma*, *Orthotylus*, and *Mecyclothorax* beetles: Rubinoff 2008; Polhemus 2011; Liebherr 2011), and is of critical importance in understanding and conserving Hawaiian biodiversity.

Cryptic sibling species

The occurrence of cryptic sibling species in *Nesophrosyne* has been previously demonstrated in the *kanawao* species group associated with *Broussaisia arguta* (Figs. 3 & 5, Clade M: Bennett and O'Grady 2011). Species in this group are morphologically indistinguishable, demonstrating high levels of local endemism and genetic divergence. This pattern is repeated among *Nesophrosyne* clades specializing on other widespread host-plants, occurring in nearly every clade (Fig. 3), including A (*Pipturus*), D (*Myoporum*) E, (*Dodonaea*), G (*Hedyotis* and *Coprosma*), I (*Coprosma*), K (*Melicope*), M (*Broussaisia*), and N (*Psychotria* and *Clermontia*). Cryptic species groups comprise sibling species distributed between islands and volcanoes, generally restricted to plant lineages. The existence of cryptic species has been established for few other Hawaiian groups, especially to this degree and scale (but see Mendelson and Shaw 2005). To our knowledge, the presence of replicated clades of cryptic species, as a result of the confluence of ecological and biogeographic processes, has not been demonstrated among other leafhopper genera. Undoubtedly, cryptic sibling species are much more common than currently understood in cicadellids and Hawaiian insects.

Several operating forces may drive the formation and maintenance of cryptic groups on island archipelagoes, including maintained selection for host-plant specialization across a geographic range, and behavioral mating cues (Bickford et al. 2006). Specialization on host-plants requires complex morphological, behavioral, and physiological adaptations to establish

host preference and to overcome host defenses (Schoonhoven 2004; Futuyma and Agrawal 2009; Denno and Kaplan 2007). Thus, selection to maintain host specialization can sustain morphological stasis in lineages that have secondarily dispersed to different islands or volcanoes, but occupy the same fundamental niche (Bickford et al. 2006; Schonogge et al. 2002). On the other hand, behavioral selection on non-visual mating cues can also sustain morphological stasis in speciating lineages by creating rapid and lasting barriers between established populations, requiring little or no morphological change (Henry 1994). This pattern has been documented in other hemipteroid groups, like *Nesophrosyne*, which use abdominal membranes to sing (Henry 1994; Sueur and Puissant 2007). While undoubtedly important, further study is required to tease apart sexual mechanisms in *Nesophrosyne* diversification.

Host-plant use in Nesophrosyne

Osborn (Osborn 1935) noted the importance of the native Hawaiian flora in forming ecological opportunities for established native herbivorous insects. *Nesophrosyne* species are highly host-plant specific and have diversified extensively among endemic dicotyledons, which is unique in the leafhopper subfamily Deltocephalinae that is largely known for associations with grasses and sedges (Zahniser and Dietrich 2010). Patterns observed in *Nesophrosyne* are consistent with what is known about other endemic Hawaiian herbivorous insect lineages. The genus *Nesosydne* (Delphacidae) demonstrates similarly high host-plant specificity, and has radiated among 28 plant families from a hypothesized monocot-restricted ancestor (Roderick and Percy 2008; Roderick 1997). The genus *Orthotylus* (Miridae) contains many highly host-plant specific species, utilizing at least 16 plant families (Polhemus 2011). Other mirid genera, *Sarona*, *Nesiomiris*, and *Cyrtolepis*, are all thought to have expanded their host breadth from a host specific origin (reviewed in Roderick and Percy 2008).

The broad patterns of host-plant specialization are complex in Hawaiian herbivores, resulting from a complicated interplay between host-plant specialization, host-plant transitions, and island geography. Host specialization requires morphological, behavioral, and physiological adaptations in response to plant defenses, morphology, chemistry, inter-specific interactions with other insect groups, and local environmental conditions, requiring careful study to disentangle (reviewed in Schoonhoven et al. 2005; Futuyma and Agrawal 2009; Denno and Kaplan 2007). Unfortunately, the interplay between host-plant use and island geography remains unclear with the lack of resolution of many of the endemic plant and insect lineages for comparison with results presented here (Roderick and Percy 2008). Despite this situation, four dominant patterns emerge from species diversification and host specialization of *Nesophrosyne* that can provide novel insights into Hawaiian herbivore evolution: 1) diversification through host-plant switching; 2) allopatric diversification within a host-plant lineage; 3) repeated, independent shifts to the same host-plant lineages; 4) and, absence or low abundance on some host-plant lineages.

Diversification through host-plant switching

Transitions to different native host-plant families, genera, and species play a significant role in the diversification and distribution of *Nesophrosyne* species. *Nesophrosyne* shows little evidence of being broadly restricted to evolutionarily similar plant lineages as seen in some other island groups (*e.g.,* psyllids and *Epicephala* moths: Percy et al. 2004; Hembry et al. 2011), or to have coevolved with particular host taxa (Thompson 1989). Instead, host-plant switching in

Nesophrosyne operates across geographic and temporal scales, and at the clade and species levels (Figs. 3-5). Host shifting can result in dramatic and rapid species diversification, extensive morphological diversity, and the expansion of species' biogeographic distributions (Cocroft et al. 2008) as seen in *Nesophrosyne*.

Percy et al. (2004) posited three potential factors that govern host-plant switching in island taxa: 1) open niche space, 2) host-plant abundance, and 3) geographic proximity of host lineages. Oceanic islands are formed *in situ* and therefore present successfully colonizing lineages with open dynamic niche space (Wagner and Funk 1995). Over time, these landscapes evolve into lush species-rich forests, providing a diverse array of potential host-plants for radiating insect herbivores. The endemic Hawaiian flora has arrived periodically throughout the formation of the islands, radiating into exceptionally diverse groups that have secondarily lost dispersal abilities and plant defenses, thus providing novel opportunities for insect adaptation (Givnish 1998; Price and Clague 2002). This dynamic landscape of host niches presents unparalleled opportunity for ecological release in island insects, and endemic herbivores have routinely capitalized on this opportunity (Roderick and Percy 2008).

Host-plant abundance and proximity are both important factors in the diversification of *Nesophrosyne*. Species have diversified among some of the most dominant plant families, genera, and species with widespread distributions throughout Hawai`i (*e.g., Psychotria, Coprosma, Broussaisia*: Wagner et al. 1999). For example, many of the basal Kaua`i lineages demonstrate localized host switching among co-occurring, locally dominant plant genera in the plant family Urticaceae (Figs. 3 & 4, Clade A) and between seven proximally abundant host-plant families (Fig. 3, Clade B). Similar patterns occur extensively among locally abundant plant groups on the younger island, O`ahu (Fig. 3, Clades D & N). On the youngest island, Hawai`i, multiple clades demonstrate local host shifts between ecologically dominant hosts, including Clade N (shifts from *Psychotria* to *Clermontia*, which co-occur in similar habitats) and Clade C (host transitions between five plant genera each in different families). The repeated pattern of host-plant transitions on both old and young islands, and especially across large evolutionary distances between hosts, indicates that this pattern is a continually operating driver of diversification in *Nesophrosyne*.

Specialization on widespread host lineages and allopatric speciation

Osborn (1935) predicted that *Nesophrosyne* lineages initially adapt to novel hosts, and subsequently diversify in allopatry according to island geography. Under this hypothesis, species specializing on the same host lineage throughout multiple islands are expected to be closely related and was found among the *kanawao* species group associated with *Broussaisia arguta* (Bennett and O'Grady 2011). This prediction is born out in other large *Nesophrosyne* clades associated with widespread, monotypic host-plant lineages, including *Dodonaea viscosa* (Clade E), *Pipturus albidus* (A), and *Myoporum sandwicense* (D). This pattern further emerges among the widely diversified host genera *Melicope* (K), *Coprosma* (I), *Psychotria* (C, F, & N), and *Myrsine* (O). This pattern highlights the important role of island geography in species diversification, as Island topography has promoted allopatric species diversification across volcanic ranges. Several clades have basally diverging lineages on younger islands (*e.g.,* Clades I & E), while others have descended down the chain from older islands in a progression-rule pattern (*e.g.,* Clades A & E: Wagner and Funk 1995), further demonstrating the ongoing, dynamic diversification of *Nesophrosyne* among the Hawaiian biota. This process apparently

requires little morphological change and has resulted in multiple clades of genetically distinct, morphologically cryptic species described above.

Repeated, independent specialization on host lineages

Nesophrosyne has transitioned and specialized on the same or similar host-plant types multiple independent times and on different islands. This pattern is observed in several widespread host lineages, including *Clermontia* spp., *Dodonaea viscosa*, and *Broussaisia arguta*, among others (Figs. 3-5, Clades N, E, M). *Nesophrosyne* species have transitioned to wet forest species in the plant genus *Clermontia* at least three independent times, each on different islands. Species specializing on *Dodonaea* and *Broussaisia* form clades spanning O`ahu through Hawai`i Island, but demonstrate independent transitions to that niche on Kaua`i. This pattern may be the result of the relative isolation of Kaua`i from other islands (approximately 128 km versus a maximum of 48 km for other inter-island distances), indicating a possible barrier limiting dispersal to Kaua`i. However, this pattern needs to be more closely examined in other groups.

The repeated, independent specialization among host-plants is analogous with the 'ecomorph' concept, which has been invoked in an adaptive radiation context to describe congeneric species that independently evolve to fill or specialize in similar ecological niches, such as the same host-plant lineages on different islands (Gillespie 2004; Yoder et al. 2010). Ecomorph patterns are also observed in the endemic Hawaiian *Tetragnatha* spider radiation (Gillespie 2004), and suggest that local adaptation is more easily accomplished to fill niches than dispersal by specialized lineages from different islands. In low dispersing groups with high levels endemicity, this pattern is expected between islands, especially if niche space is open and abundant, as are many host-plant lineages (*e.g., Dodonaea* and *Broussaisia*). Ecomorph formation in island herbivores may be facilitated by inter-specific interactions with other insects, host-plant morphology, and host chemistry (Schoonhoven et al. 2005; Futuyma and Agrawal 2009), requiring locally sustained interactions for adaptation to occur. This process would preclude dispersal from adjacent islands to fill that niche.

Absence or low abundance on host-plants

Nesophrosyne demonstrates a general absence from the plant families Asteraceae and Fabaceae, and a comparatively low abundance on Campanulaceae, the genus *Cyrtandra* (Gesnerieaceae), and *Metrosideros polymorpha*. These lineages represent some of the oldest, most species-rich, or most ecologically dominant endemic Hawaiian plant groups (Wagner et al. 1999; Givnish et al. 2009). While *Nesophrosyne* may utilize these hosts undetected, collections (or lack thereof) made here corroborate a similar pattern among historical collections of observed absence and low relative abundance when compared to specialization on other hosts (SI Table 2). Direct comparison of host-specialization patterns between *Nesophrosyne* and other endemic herbivore lineages suggests the intriguing possibility that insect-insect interactions play a large role in the exclusion of *Nesophrosyne* from some hosts.

Competition occurs between lineages requiring similar resources, with recent work suggesting that plant-mediated interactions (*e.g.,* plant defenses) can facilitate indirect competition between non-interacting organisms (reviewed in Denno and Kaplan 2007). This process can lead to the asymmetric exclusion of insects from some plant species or entire groups (Denno and Kaplan 2007). The potential for competition among Hawaiian herbivores is strong,

given that several other hemipteroid radiations have specialized broadly on overlapping host-plants. For example, *Nesophrosyne* is surprisingly rare and restricted in its association with *Metrosideros polymorpha*, one of the most ecologically dominant plants throughout the archipelago (Wagner et al. 1999). Although *Nesophrosyne* does specialize on *M. polymorpha*, it occurs in localized populations, often on only a single plant. *Metrosideros polymorpha* is one of the dominant hosts for endemic gall-forming psyllids (Nishida et al. 1980), and the mosaic exclusion of *Nesophrosyne* can be explained by the impact of gall formation, which alters leaf chemistry, protein content, and leaf morphology (Bagatto et al. 1996).

Of the endemic Hawaiian herbivore groups, only *Nesosydne* occupies the same basic niche as *Nesophrosyne* (phloem feeding, oviposition, and nymphal development: Denno and Roderick 1990), which can lead to direct competition and asymmetric exclusion of both genera from some hosts (Denno and Kaplan 2007). As predicted under this hypothesis, *Nesosydne* species have specialized broadly on the elements of the Hawaiian flora that *Nesophrosyne* is not known to occupy or has not radiated on extensively, including Asteraceae, Fabaceae, Campanulaceae, and Gesneriaceae (Zimmerman 1948; Roderick and Percy 2008). Conversely, *Nesophrosyne* has diversified on plant families that are complementary to those occupied by *Nesosydne*, including Rubiaceae, Goodeniaceae, Myrsinaceae, Rutaceae, Sapindaceae, and Hydrangeaceae, among others (Zimmerman 1948; Roderick and Percy 2008). The underlying mechanisms and the degree to which this observation holds across islands are currently unknown, but the patterns help in understanding the distribution and diversification of Hawaiian herbivores, and warrant further study.

Conclusion

Nesophrosyne is perhaps the most diverse herbivore radiations endemic to the Hawaiian Islands, and heretofore one of the least known. Results demonstrate that this exceptional diversification is due to a confluence of processes associated with host-plant specialization. In an adaptive radiation context, it is clear that the ecological opportunity offered by the dynamic diversity of host-plants, drives the diversification of specializing insects, which has remained previously unclear (Yoder et al. 2010). Host-plant associations have promoted at least three parallel processes: 1) extensive host switching, resulting in ecological and morphological diversification; 2) adaptation and retention of host lineages, resulting in extensive allopatric cryptic diversity; and 3) the parallel formation of ecomorphs. Finally, evidence suggests that competing herbivore radiations may restrict and limit ecological opportunity. The result that, in diverse herbivorous groups, multiple evolutionary processes play fundamental roles in species diversification is fundamental for understanding of how adaptive radiations proceed and how biodiversity is organized and maintained. More studies of groups like *Nesophrosyne* are required to determine the extent to which these processes govern herbivorous insect diversity globally.

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Tables

Table 1: Genomic loci used in phylogenetic analysis of *Nesophrosyne*.

Gene	Genome	# of Characters (Parsimony Informative Chars.)	Primer Name (Annealing Temp.)	Primer Sequence 5'-3'
Cytochrome Oxidase II (COII)	mtDNA	714 (434)	TL2-J-3037[¶] (53°C)	TAGTATGGCAGATTAGTCAATGAA
			C2-N-3661[¶]	CCRCAAATTTCWGARCATTGACCA
Cytochrome Oxidase I (COI)	mtDNA	675 (319)	LCO[†] (3-Step)	GCTCAACAAATCATAAAGATATTGG
			HCO[†]	TAAACTTCAGGGTGACCAAAAAATCA
Nuclear Histone 3 (H3)	nucDNA	309 (101)	H3F[§] (62°C)	ATGGCTCGTACCAAGCAGACGGC
			H3R[§]	ATATCCTTGGGCATGATGGTGAC
Wingless (wg)	nucDNA	303 (93)	wg-1.5f[¥](62°C)	GTSAARACBTGYTGGATGCG
			wg-7.5B[¥]	GTCCTGTAMCCVCGUCCACAACACAT
			wg-4F[*] (62°C)	CCGTGTGGTCGGGGACAACC
			wg-4R[*]	CCCGTCCACCCCGATGCTTG
16S	mtDNA	519 (243)	LR-J-12887[#] (53°)	CCGGTYTGAACTCARATCA
			LR-N-13398[#]	CRMCTGTTAWCAAAAACAT
16S Betaproteobacteria (CoBE-16S)	endosymbiont	1000 (245)	10F[¢] (58°)	AGTTTGATCATGGCTCAGATTG
			1507R[¢]	TACCTTGTTACGACTTCACCCCAG
			Beta_300[*] (60°)	GCGGATGTATAGCCACACTGGGA
			Beta_1352[*]	ATAAAACTTACTCCCATGGTG

[¶] Takiya *et al.* 2006
[§] Ogden and Whiting 2003
[¥] Cryan *et al.* 2004
[#] Dietrich *et al.* 1997
[†] Folmer *et al.* 1994
[¢] Munson *et al.* 1991
[*] Primers designed in this study

Table 2: Akaike Informations Criterion (AIC) summary statistics for data partitioning strategies reconstructed in RAxML v7.2.7 (Stamatakis et al. 2008).

Partitions	Partition elements	l^a	K[b]	AIC[c]	ΔAIC[d]	w^e	Rank[f]
1	Concatenated	-110374.19	434	221616.39	7838.40	0	10
2	Gene type	-109814.63	443	220515.27	6737.28	0	9
3	Genome	-108662.27	452	218228.55	4450.55	0	8
4	Universal codons and single ribosome	-108413.90	461	217749.80	3971.80	0	7
5	Universal codons, 16S, and CoBE-16S	-107825.71	470	216591.43	2813.44	0	5
6	Individual gene	-108291.86	479	217541.72	3763.73	0	6
9	Individual gene codons 1+2, 3 and single ribosome	-107188.59	506	215389.18	1611.18	0	4
10	Individual gene codons 1+2, 3, 16S, and CoBE-16S	-106729.23	515	214488.47	710.48	5.257E-155	2
13	Individual gene codons and single ribosome	-106926.69	542	214937.39	1159.40	1.7347E-252	3
14	Individual gene codons, 16S, and CoBE-16S	-106337.99	551	213777.99	0	1	1

[a] log likelihood (lnL) of the ML phylogenetic hypotheses under each partitioning scheme
[b] Number parameters = # of branch lengths (*2n-3; n = taxa*) plus 9 parameters for each implementation of the GTR+GAMMA model.
[c] $AIC = -2(inL) + 2K$
[d] $\Delta AIC = AIC - AIC_{min}$
[e] AIC weight estimated as the relative likelihood = $exp(-0.5 \times \Delta AIC) / \Sigma exp(-0.5 \times \Delta AIC)$
[f] Rank based on ΔAIC

Table 3: Summary of nucleotide models determined for individual partitions using the BIC in Modeltest (Posada and Crandal 1998). Implemented models are the closest nucleotide models incorporating the necessary parameters and settings applied in MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003).

Gene	Codon Partition	BIC	Implemented model
COII	1st	TrN+I+G	GTR+I+G
	2nd	TVM+I+G	GTR+I+G
	3rd	GTR+I+G	GTR+I+G
COI	1st	Sym+I+G	GTR+I+G (Modified)
	2nd	TrN+G	GTR+G
	3rd	GTR+G	GTR+G
H3	1st	TrNef	GTR (Modified)
	2nd	JC	JC
	3rd	TVM+G	GTR + G
wg	1st	JC+G	JC+G
	2nd	JC	JC
	3rd	TVM+I+G	GTR + I + G
16S	-	TrN+I+G	GTR+I+G
CoBE-16S	-	GTR+I+G	GTR+I+G

Figures

Figure 1: Native Hawaiian leafhopper species (Cicadellidae: *Nesophrosyne*). Species names from top left to right: *"N. sp.125"*, "*N. sp.253"*, *N. sp.296"*, *N. anguilifera*, and *N. cinera*; bottom left to right: *N. craterigena* , "*N. sp.23"*, *N. oreadis*, *N. notatula*, and *"N. sp.293"*.

Figure 2: Bayesian phylogram for fully partitioned analyses, highlighting relationships among taxa of tribe Opsiini (in blue boxes) and other members of the Deltocephalinae subfamily. See SI Table 1 for collection locality information. Inset phylogeny shows placement of the featured genera in the full phylogeny. Lineages within *Nesophrosyne* are collapsed here, and presented in detail in Fig. 3. *Deltocephalus* sp. is designated as the outgroup. Numbers above and below the branches represent nodal posterior probabilities and ML bootstrap support values, respectively.

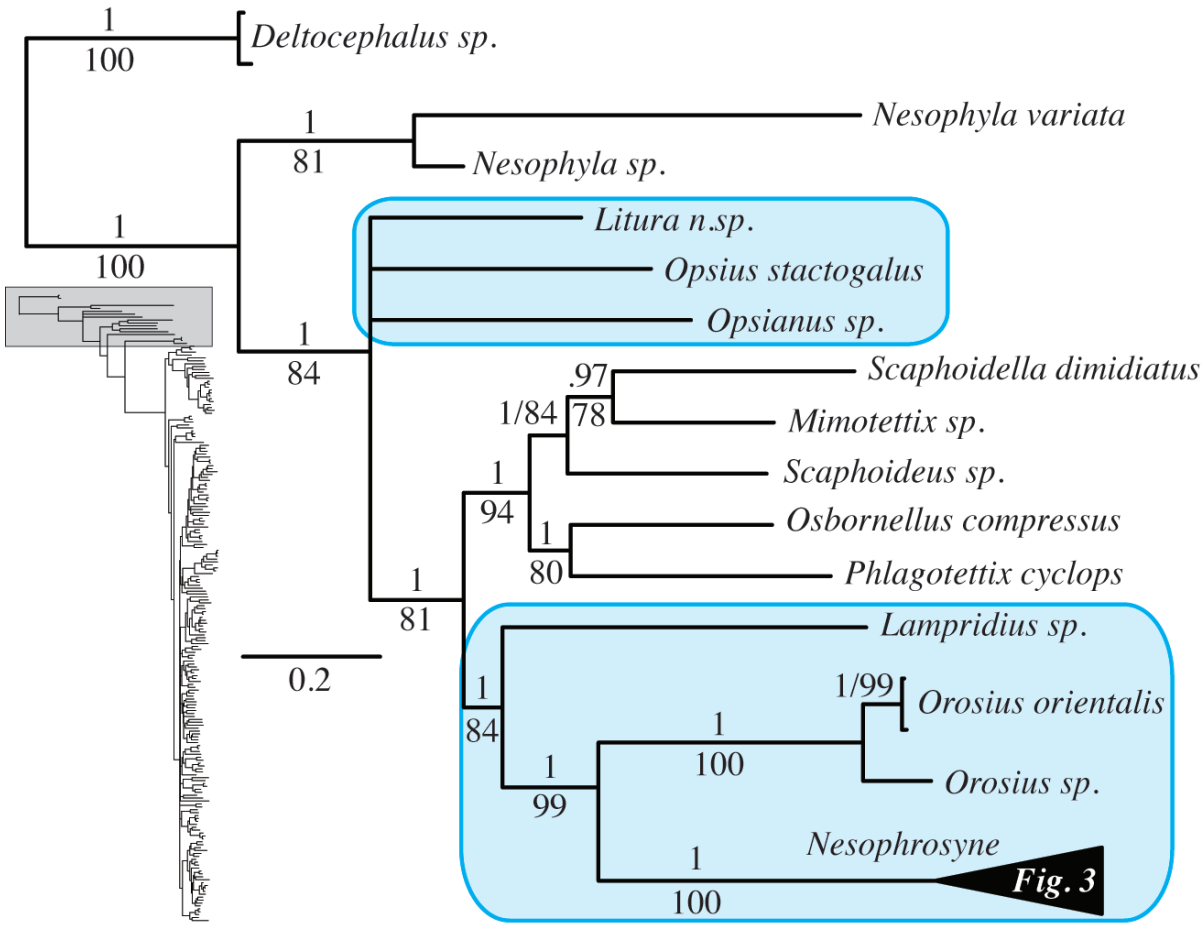
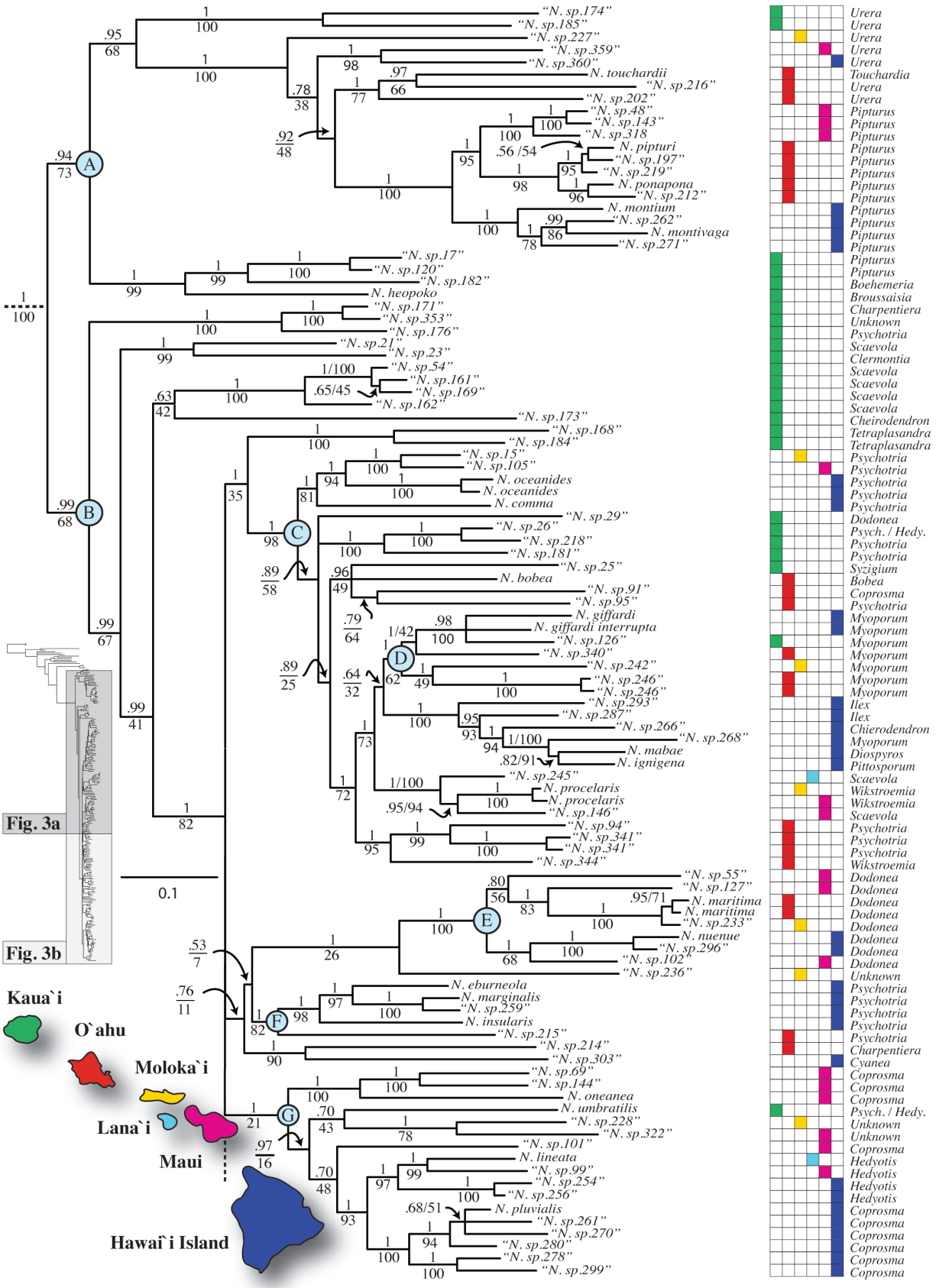


Figure 3: Bayesian phylogram for the fully partitioned analyses, showing the ingroup relationships for 191 *Nesophrosyne* species across 198 individuals (SI Table 1 for species information). Ingroup topology is split into A and B (see inset phylogeny), and outgroups have been removed (see Fig. 2). Nodes with circled letters refer to clades discussed in the text. The color-coded grid specifies the islands of endemicity for each taxon and correspond to the colored Hawaiian Islands. Dominant host-plant (*Genus*) for each species is given adjacent to cells. Numbers above and below the branches represent nodal posterior probabilities and ML bootstrap support values, respectively.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"N. sp.359"
"N. sp.360"
N. touchardii
"N. sp.216"
"N. sp.202"
"N. sp.48"
"N. sp.143"
"N. sp.318"
N. pipturi
"N. sp.197"
"N. sp.219"
N. ponapona
"N. sp.212"
N. montium
"N. sp.262"
N. montivaga
"N. sp.271"
"N. sp.17"
"N. sp.120"
"N. sp.182"
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"N. sp.162"
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"N. sp.168"
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N. oceanides
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"N. sp.218"
"N. sp.181"
"N. sp.25"
N. bobea
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N. giffardi
N. giffardi interrupta
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N. ignigena
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N. procelaris
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N. maritima
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N. insularis
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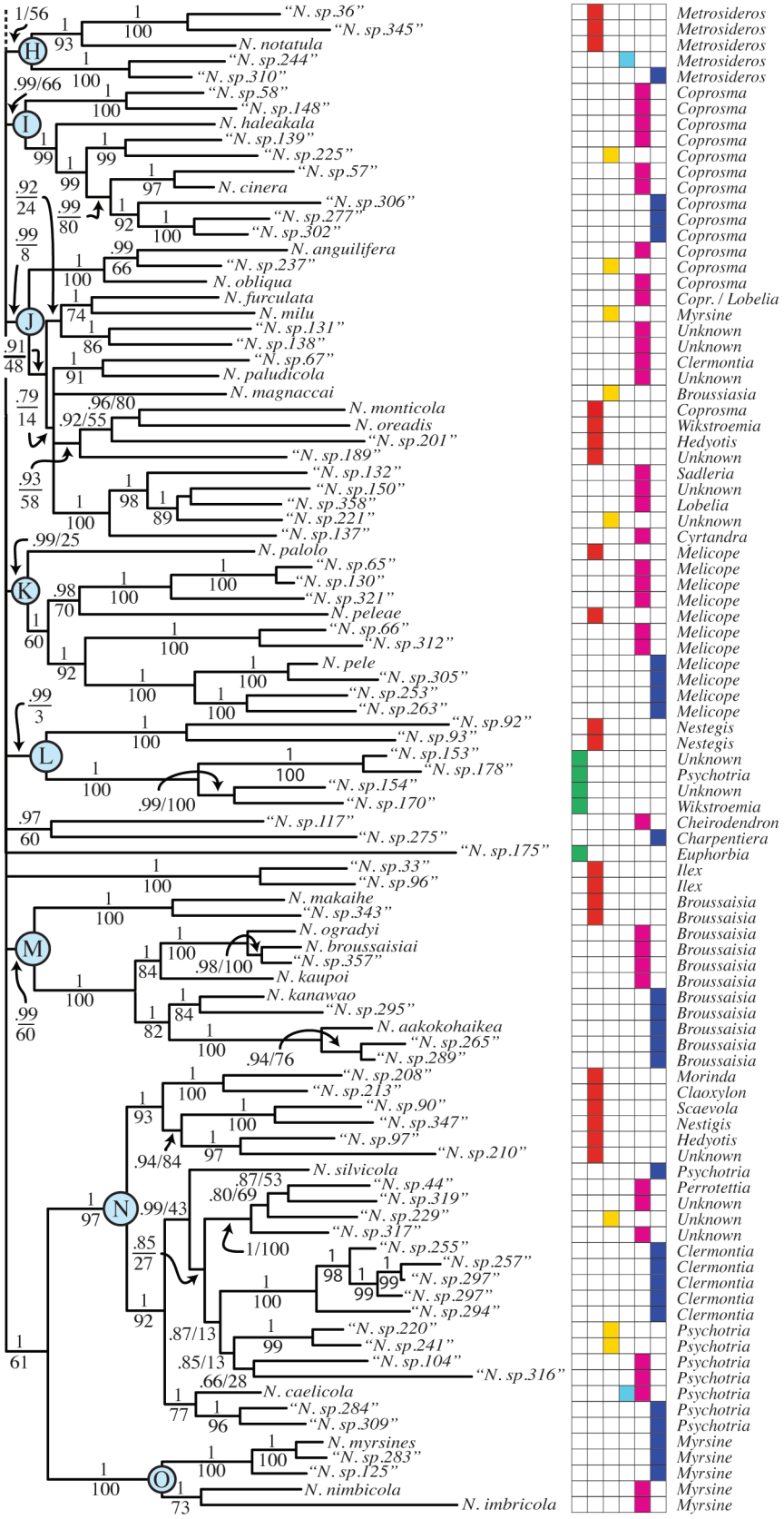
"N. sp.299"
A
B
C
D
E
F
G
Fig. 3a
Fig. 3b
0.1
Kaua`i
O`ahu
Moloka`i
Lana`i
Maui
Hawai`i Island
Urera
Urera
Urera
Urera
Urera
Touchardia
Urera
Urera
Pipturus
Pipturus
Pipturus
Pipturus
Pipturus
Pipturus
Pipturus
Pipturus
Pipturus
Pipturus
Pipturus
Pipturus
Pipturus
Pipturus
Pipturus
Pipturus
Boehemeria
Broussaisia
Charpentiera
Unknown
Psychotria
Scaevola
Clermontia
Scaevola
Scaevola
Scaevola
Scaevola
Cheirodendron
Tetraplasandra
Tetraplasandra
Psychotria
Psychotria
Psychotria
Psychotria
Psychotria
Psychotria
Dodonea
Psych. / Hedy.
Psychotria
Psychotria
Syzigium
Bobea
Coprosma
Psychotria
Myoporum
Myoporum
Myoporum
Myoporum
Myoporum
Myoporum
Myoporum
Myoporum
Ilex
Ilex
Chierodendron
Myoporum
Diospyros
Pittosporum
Scaevola
Wikstroemia
Wikstroemia
Scaevola
Psychotria
Psychotria
Psychotria
Wikstroemia
Dodonea
Dodonea
Dodonea
Dodonea
Dodonea
Dodonea
Dodonea
Dodonea
Dodonea
Unknown
Psychotria
Psychotria
Psychotria
Psychotria
Psychotria
Charpentiera
Cyanea
Coprosma
Coprosma
Coprosma
Psych. / Hedy.
Unknown
Unknown
Coprosma
Hedyotis
Hedyotis
Hedyotis
Hedyotis
Coprosma
Coprosma
Coprosma
Coprosma
Coprosma
Coprosma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N. anguilifera
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N. obliqua
N. furculata
N. milu
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"N. sp.67"
N. paludicola
N. magnaccai
N. monticola
N. oreadis
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"N. sp.175"
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N. ogradyi
N. broussaisiai
"N. sp.357"
N. kaupoi
N. kanawao
"N. sp.295"
N. aakokohaikea
"N. sp.265"
"N. sp.289"
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N. myrsines
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N. nimbicola
N. imbricola
H
I
J
K
L
M
N
O
Metrosideros
Metrosideros
Metrosideros
Metrosideros
Metrosideros
Coprosma
Coprosma
Coprosma
Coprosma
Coprosma
Coprosma
Coprosma
Coprosma
Coprosma
Coprosma
Coprosma
Coprosma
Coprosma
Copr. / Lobelia
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Clermontia
Unknown
Broussiasia
Coprosma
Wikstroemia
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Unknown
Sadleria
Unknown
Lobelia
Unknown
Cyrtandra
Melicope
Melicope
Melicope
Melicope
Melicope
Melicope
Melicope
Melicope
Melicope
Melicope
Melicope
Nestegis
Nestegis
Unknown
Psychotria
Unknown
Wikstroemia
Cheirodendron
Charpentiera
Euphorbia
Ilex
Ilex
Broussaisia
Broussaisia
Broussaisia
Broussaisia
Broussaisia
Broussaisia
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Unknown
Unknown
Unknown
Clermontia
Clermontia
Clermontia
Clermontia
Clermontia
Psychotria
Psychotria
Psychotria
Psychotria
Psychotria
Psychotria
Psychotria
Myrsine
Myrsine
Myrsine
Myrsine
Myrsine

Figure 4: Selected clades from *Nesophrosyne* Bayesian phylogenetic tree shown in Fig. 3. Clade A demonstrates host transitions between four closely related endemic plant genera in the family Urticaceae, and a single transition to the host family Hydrangeaceae. Clade N demonstrates host transitions across multiple distantly related, endemic host-plant families. Clade O is strictly associated with *Myrsine* (Myrsinaceae).

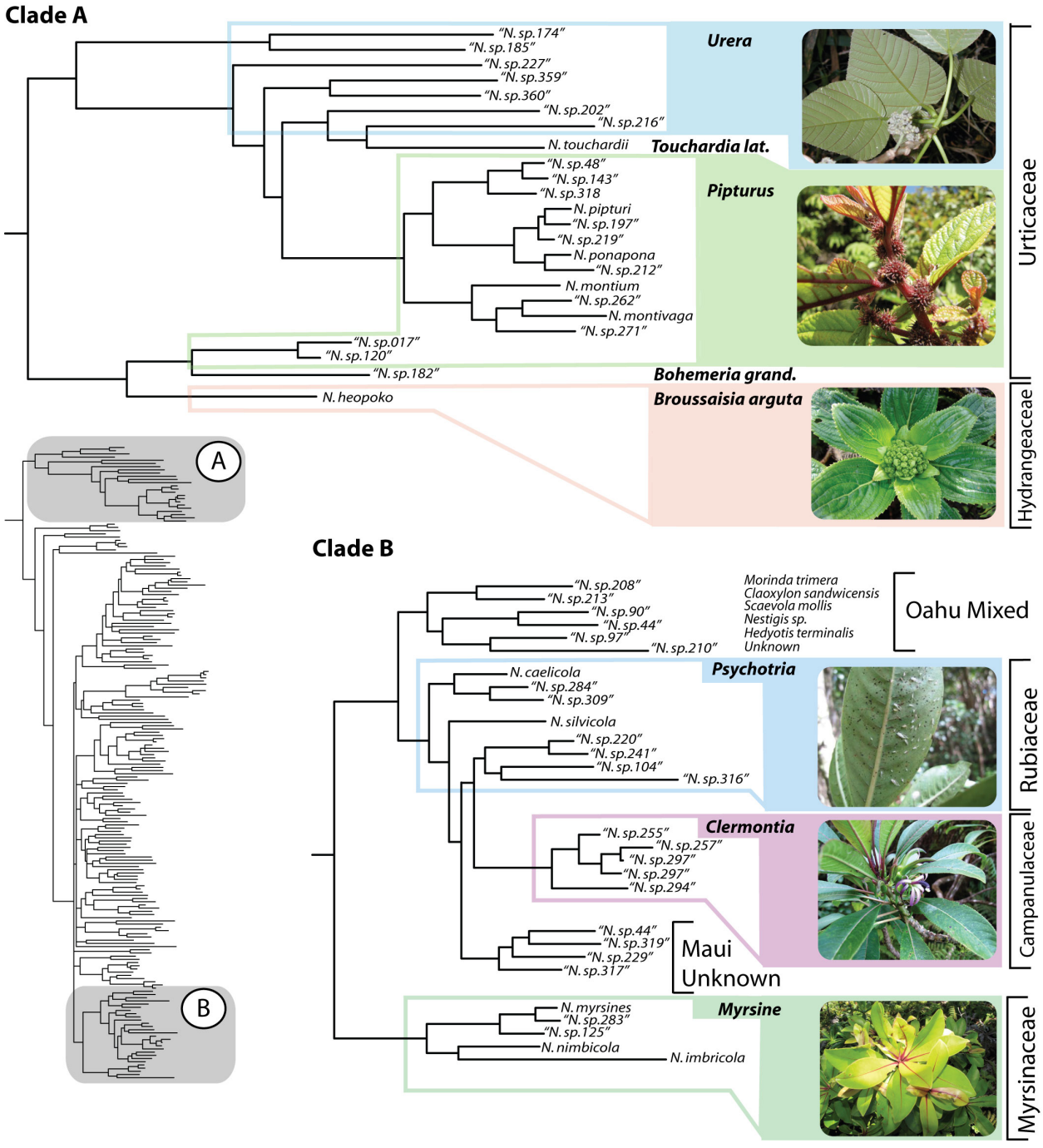
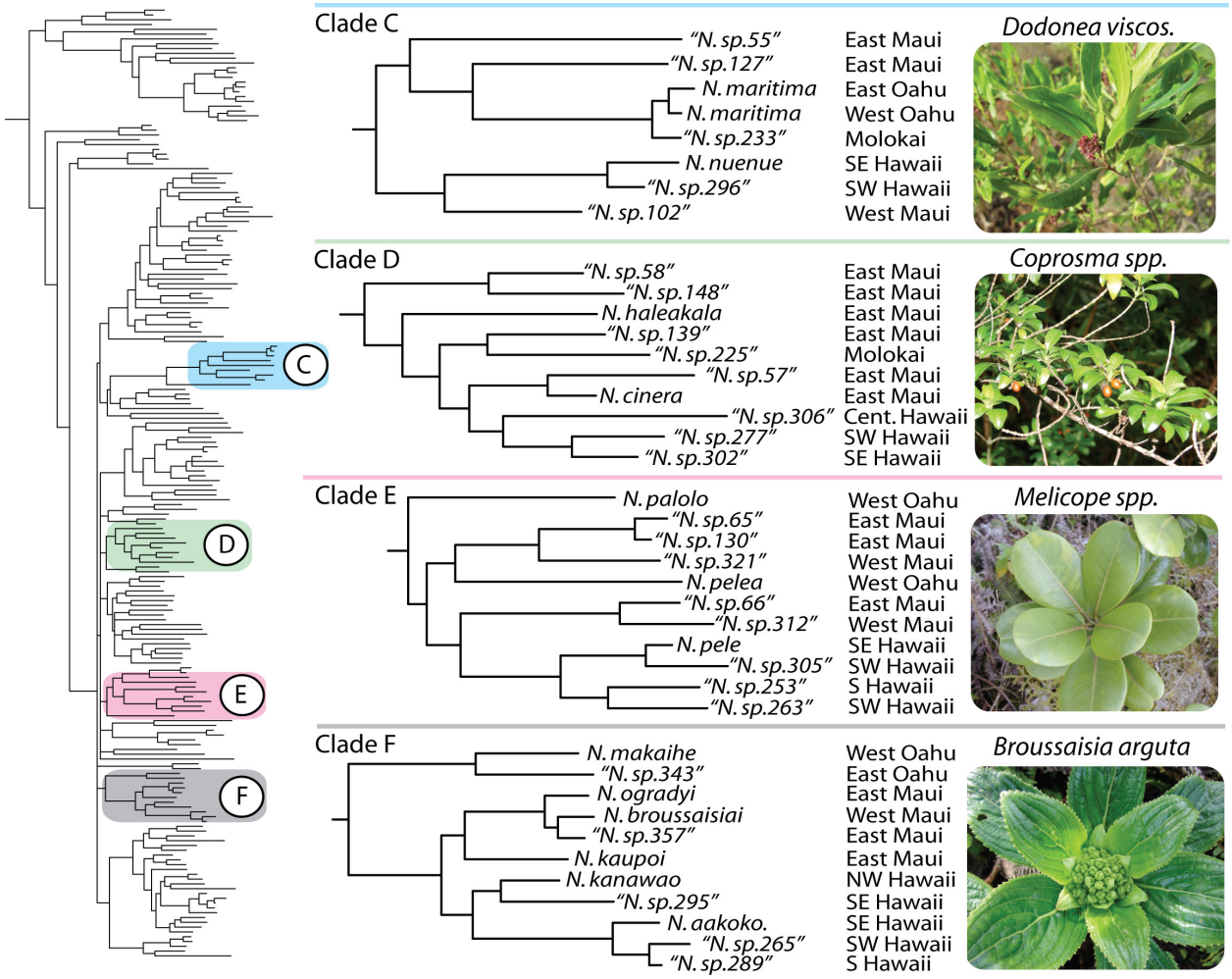
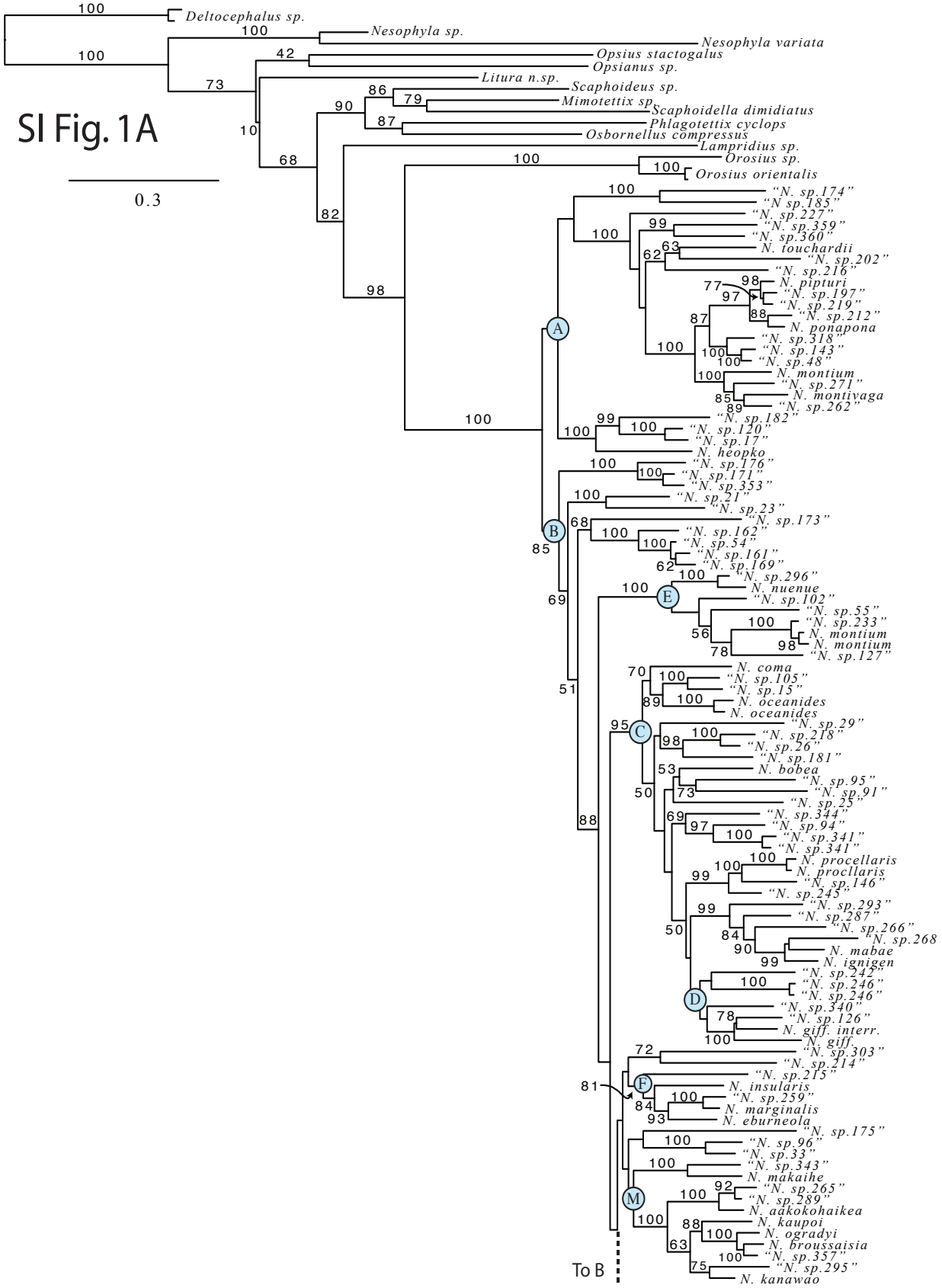


Figure 5: Selected clades from *Nesophrosyne* Bayesian phylogenetic tree shown in Fig. 3. Clades demonstrate monophyletic assemblages of species specializing on widespread host-plant lineages throughout multiple Hawaiian Islands. Island and area of endemicity for each leafhopper species is given. Islands are sub-divided according to region. Abbreviations for Hawai`i Island correspond to relative compass direction of each region: N = North, South = South, NW = North West, SW = South West, SE = South East, Cent. = Central.



Supplementary Information

Supplementary Figure 1: Maximum Likelihood phylogram for the fully partitioned analyses with the betaproteobacterial 16S (CoBE-16S) locus removed. The tree shows the ingroup relationships for 191 *Nesophrosyne* species across 198 individuals (SI Table 1 for species information) and outgroups. Topology is split into A and B. Nodes with blued-circled letters refer to clades discussed in the text (see Fig. 3 also). Numbers associated with internal branches represent nodal bootstrap support (BS) values. Nodes with BS values <50 are not shown.


SI Fig. 1A
0.3
Deltocephalus sp.
Nesophyla sp.
Nesophyla variata
Opsius stactogalus
Opsianus sp.
Litura n.sp.
Scaphoideus sp.
Mimotettix sp.
Scaphoidella dimidiatus
Phlagotettix cyclops
Osbornellus compressus
Lampridius sp.
Orosius sp.
Orosius orientalis
"N. sp.174"
"N sp.185"
"N. sp.227"
"N. sp.359"
"N. sp.360"
N. touchardii
"N. sp.202"
"N. sp.216"
N. pipturi
"N. sp.197"
"N. sp.219"
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N. aakokohaikea
N. kaupoi
N. ogradyi
N. broussaisia
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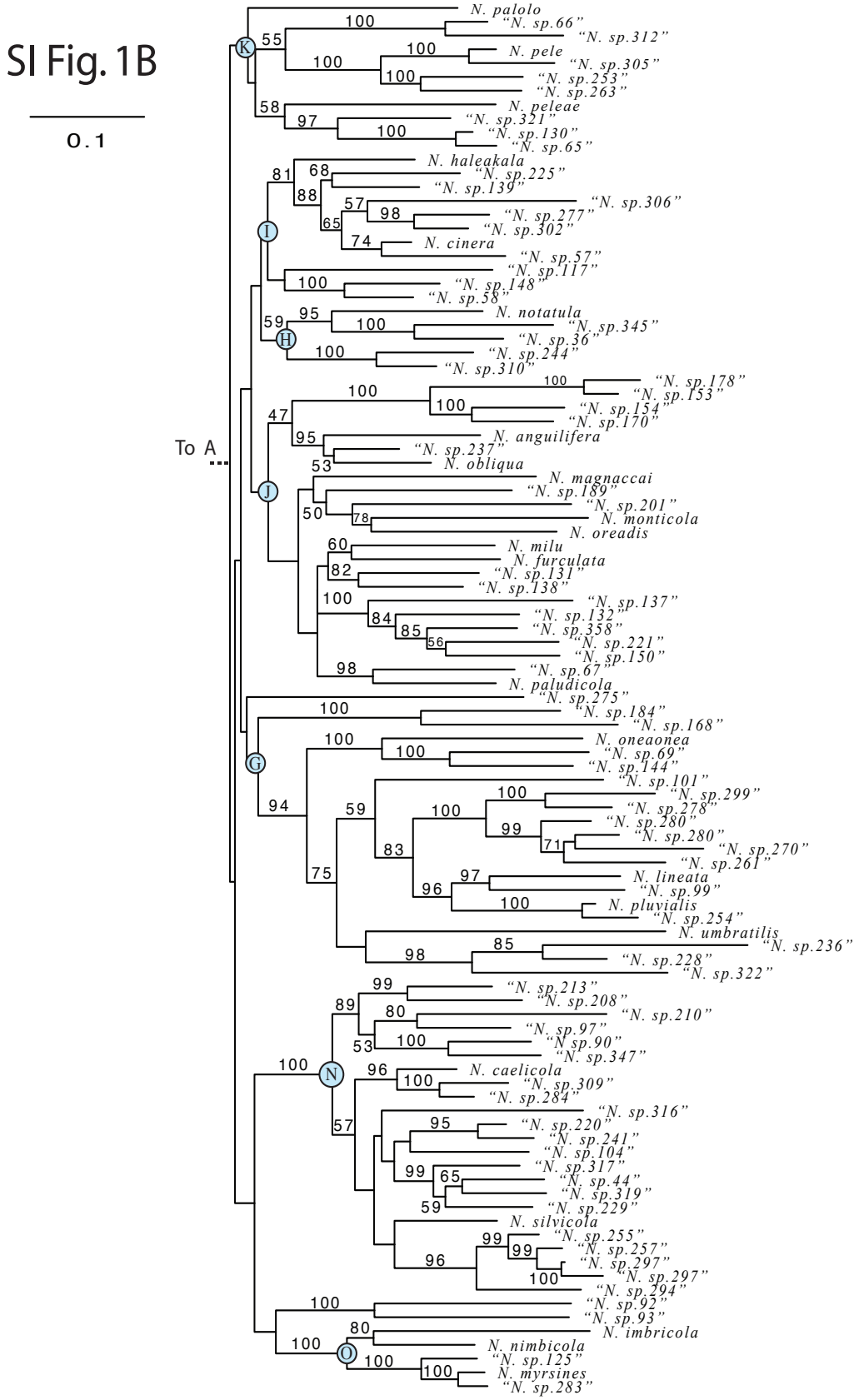
"N. sp.295"
N. kanawao
A
B
C
D
E
F
M
To B

SI Fig. 1B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To A
K
I
H
J
G
N
O
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"N. sp.306"
"N. sp.277"
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"N. sp.117"
"N. sp.148"
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"N. sp.178"
"N. sp.153"
"N. sp.154"
"N. sp.170"
N. anguilifera
"N. sp.237"
N. obliqua
N. magnaccai
"N. sp.189"
"N. sp.201"
N. monticola
N. oreadis
N. milu
N. furculata
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"N. sp.138"
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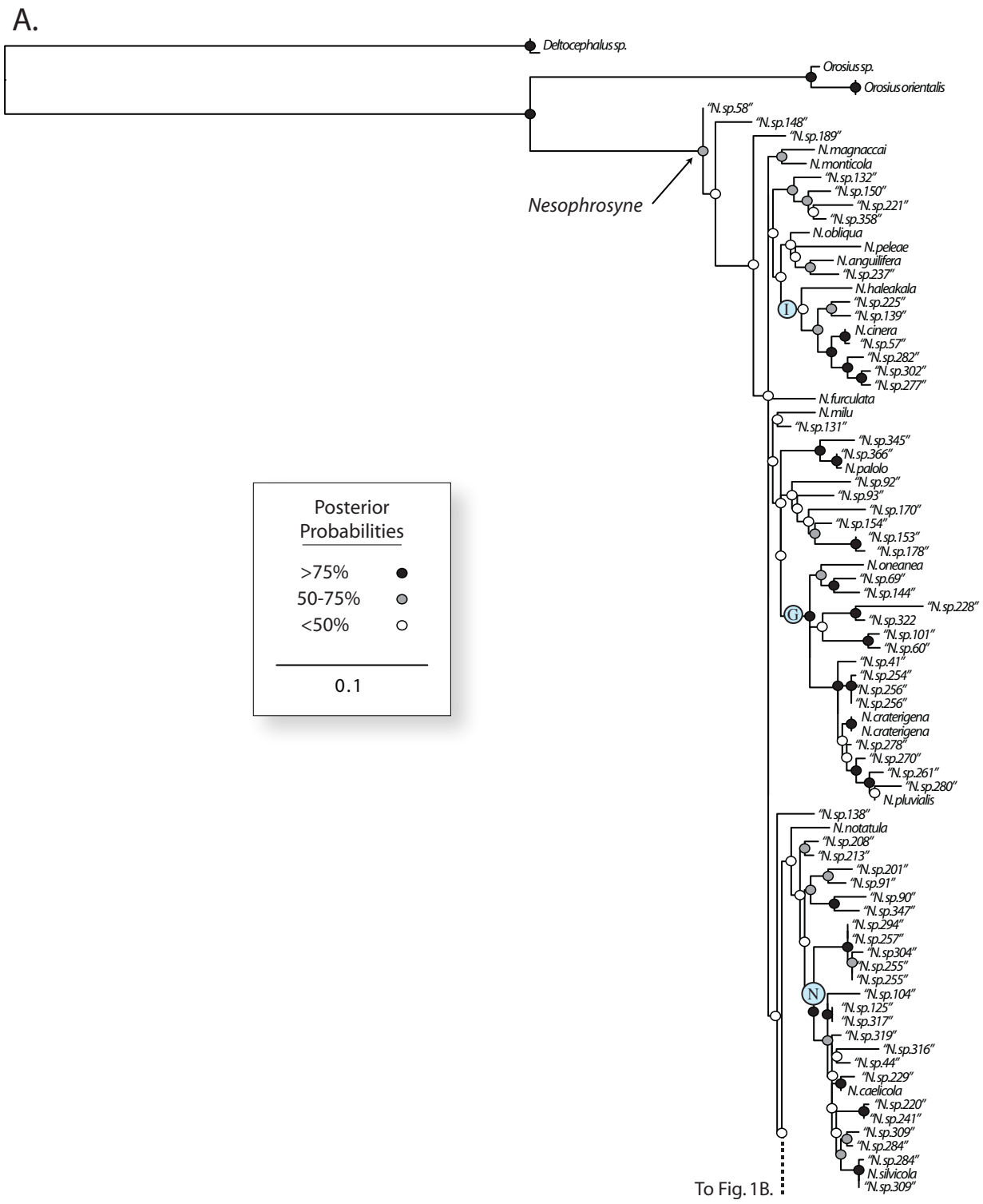
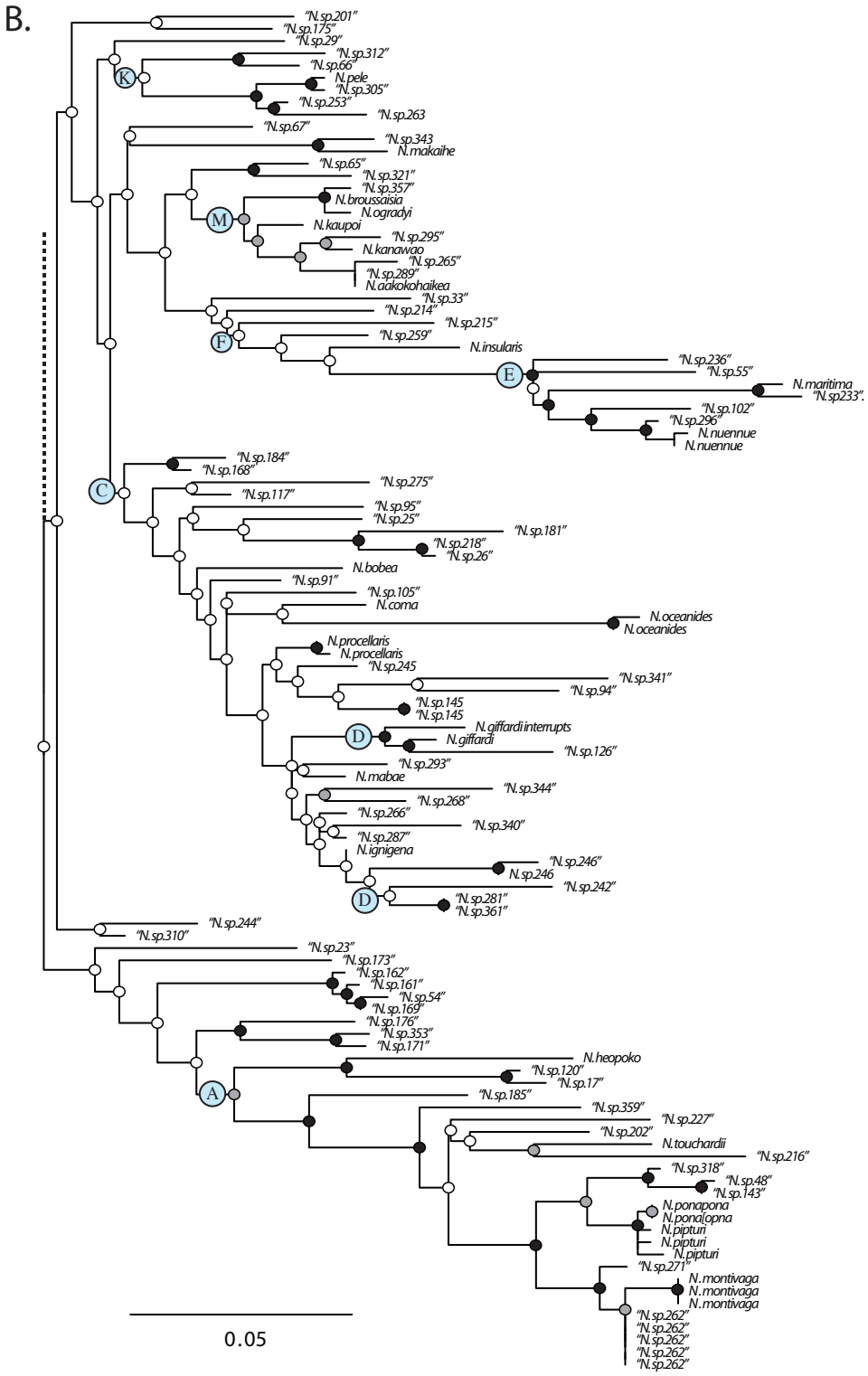
N. oneaonea
"N. sp.69"
"N. sp.144"
"N. sp.101"
"N. sp.299"
"N. sp.278"
"N. sp.280"
"N. sp.280"
"N. sp.270"
"N. sp.261"
N. lineata
"N. sp.99"
N. pluvialis
"N. sp.254"
N. umbratilis
"N. sp.236"
"N. sp.228"
"N. sp.322"
"N. sp.213"
"N. sp.208"
"N. sp.210"
"N. sp.97"
"N sp.90"
"N. sp.347"
N. caelicola
"N. sp.309"
"N. sp.284"
"N. sp.316"
"N. sp.220"
"N. sp.241"
"N. sp.104"
"N. sp.317"
"N. sp.44"
"N. sp.319"
"N. sp.229"
N. silvicola
"N. sp.255"
"N. sp.257"
"N. sp.297"
"N. sp.297"
"N. sp.294"
"N. sp.92"
"N. sp.93"
N. imbricola
N. nimbicola
"N. sp.125"
N. myrsines
"N. sp.283"

Supplementary Figure 2: Maximum Likelihood phylogram for the betaproteobacterial 16S (CoBE-16S) locus. The tree shows the relationship for the betaproteobacterial of 174 *Nesophrosyne* species in 190 individuals (see SI Table 1 for species sampling information) and 5 outgroup taxa. Global topology is split into A and B. Nodes with blue-circled letters refer to clades discussed in the text and correspond to species groups recovered in the total data alignment and dataset minus the CoBE-16S locus (see Fig. 3 and SI Fig. 1, respectively). Nodal support are represented as colored circles for clarity; see inset legend for corresponding values. **Note**: Taxonomic relationships, while receiving poor support for some clades in the single gene tree presented here (*e.g.,* Clades C, D, etc.), are congruent with the likelihood and Bayesian analyses for the entire matrix including all loci shown in Fig. 3 (see monophyletic clades labeled in blue). The same relationships are also found in the data matrix that excludes the CoBE-16S locus shown in SI Fig 1. The betaproteobacterial lineage harbored by *Nesophrosyne* is related to the "*Candidatus* Zinderia insecticola" endosymbiont of spittlebugs, which are also members of the Cicadomorpha infraorder (Bennett unpub; McCutcheon and Moran 2010). These results provide evidence for the congruent evolutionary history of the betaproteobacteria endosymbiont and bacterial host, found in other auchenhorrynchan systems (Takiya et al. 2006; Moran et al. 2008; Andersen et al. 2010; McCutcheon and Moran 2010).


A.
Deltocephalus sp.
Orosius sp.
Orosius orientalis
"N. sp.58"
"N. sp.148"
"N. sp.189"
N. magnaccai
N. monticola
"N. sp.132"
"N. sp.150"
"N. sp.221"
"N. sp.358"
Nesophrosyne
N. obliqua
N. peleae
N. anguilifera
"N. sp.237"
N. haleakala
I
"N. sp.225"
"N. sp.139"
N. cinera
"N. sp.57"
"N. sp.282"
"N. sp.302"
"N. sp.277"
N. furculata
N. milu
"N. sp.131"
"N. sp.345"
"N. sp.366"
N. palolo
"N. sp.92"
"N. sp.93"
"N. sp.170"
"N. sp.154"
"N. sp.153"
"N. sp.178"
N. oneanea
"N. sp.69"
"N. sp.144"
"N. sp.228"
G
"N. sp.322
"N. sp.101"
"N. sp.60"
"N. sp.41"
"N. sp.254"
"N. sp.256"
"N. sp.256"
N. craterigena
N. craterigena
"N. sp.278"
"N. sp.270"
"N. sp.261"
"N. sp.280"
N. pluvialis
"N. sp.138"
N. notatula
"N. sp.208"
"N. sp.213"
"N. sp.201"
"N. sp.91"
"N. sp.90"
"N. sp.347"
"N. sp.294"
"N. sp.257"
"N. sp.304"
"N. sp.255"
"N. sp.255"
N
"N. sp.104"
"N. sp.125"
"N. sp.317"
"N. sp.319"
"N. sp.316"
"N. sp.44"
"N. sp.229"
N. caelicola
"N. sp.220"
"N. sp.241"
"N. sp.309"
"N. sp.284"
"N. sp.284"
N. silvicola
"N. sp.309"
To Fig. 1B.
Posterior Probabilities
>75%
50-75%
<50%
0.1


B.
"N.sp.201"
"N.sp.175"
"N.sp.29"
"N.sp.312"
"N.sp.66"
N.pele
"N.sp.305"
"N.sp.253"
"N.sp.263
"N.sp.67"
"N.sp.343
N.makaihe
"N.sp.65"
"N.sp.321"
"N.sp.357"
N.broussaisia
N.ogradyi
N.kaupoi
"N.sp.295"
N.kanawao
"N.sp.265"
"N.sp.289"
N.aakokohaikea
"N.sp.33"
"N.sp.214"
"N.sp.215"
"N.sp.259"
N.insularis
"N.sp.236"
"N.sp.55"
N.maritima
"N.sp.233".
"N.sp.102"
"N.sp.296"
N.nuennue
N.nuennue
"N.sp.184"
"N.sp.168"
"N.sp.275"
"N.sp.117"
"N.sp.95"
"N.sp.25"
"N.sp.181"
"N.sp.218"
"N.sp.26"
N.bobea
"N.sp.91"
"N.sp.105"
N.coma
N.oceanides
N.oceanides
N.procellaris
N.procellaris
"N.sp.245
"N.sp.341"
"N.sp.94"
"N.sp.145
"N.sp.145
N.giffardi interrupts
N.giffardi
"N.sp.126"
"N.sp.293"
N.mabae
"N.sp.344"
"N.sp.268"
"N.sp.266"
"N.sp.340"
"N.sp.287"
N.ignigena
"N.sp.246"
N.sp.246
"N.sp.242"
"N.sp.281"
"N.sp.361"
"N.sp.244"
"N.sp.310"
"N.sp.23"
"N.sp.173"
"N.sp.162"
"N.sp.161"
"N.sp.54"
"N.sp.169"
"N.sp.176"
"N.sp.353"
"N.sp.171"
N.heopoko
"N.sp.120"
"N.sp.17"
"N.sp.185"
"N.sp.359"
"N.sp.227"
"N.sp.202"
N.touchardii
"N.sp.216"
"N.sp.318"
"N.sp.48"
"N.sp.143"
N.ponapona
N.pona[opna
N.pipturi
N.pipturi
N.pipturi
"N.sp.271"
N.montivaga
N.montivaga
N.montivaga
"N.sp.262"
"N.sp.262"
"N.sp.262"
"N.sp.262"
"N.sp.262"
K
M
F
E
C
D
D
A
0.05

Supplementary Table 1: Taxon sampling for non-native Hawaiian leafhoppers and *Nesophrosyne* included in molecular analyses. Taxon sampling information includes geographic area of origin, current taxonomic classification (tribe), dominant host-plant (Family: *Genus species*), collection locality, and GenBank accession numbers (percent gene coverage at bottom). Tribal designations for non-Hawaiian endemic leafhoppers follow Zahniser and Dietrich (2010) and Dai et al. (2010).

Geographic Area	*Genus species* (Tribe)	Dominant Host Plant		Collection Locality	COII	COI	H3	wg	16S	CoBE-16s
		Family	*Genus species*							
Non-native Hawaiian Leafhoppers										
So. Pacific	*Deltocephalus sp.* (Deltocephalini)	Unknown	Unknown	Moorea, French Polynesia	JX417705	JX433225	JX433638	JX433847	JX433429	JX434023
Hawai`i	*Deltocephalus sp.* (Deltocephalini)	Unknown	Unknown	Kauai, Kahuku Tr.	-	JX433226	-	JX433848	-	JX434024
So. Pacific	*Nesophyla variata* (Macrostelini)	Unknown	Unknown	Moorea, French Polynesia	JX417681	JX433206	JX433616	-	JX433405	-
So. Pacific	*Nesophyla sp.* (Macrostelini)	Unknown	Unknown	Moorea, French Polynesia	JX417682	JX433207	JX433617	JX433824	JX433406	-
Zambia	*Litura sp.* (Opsiini)	Unknown	Unknown	Zmbezi, Zambia	JX417686	JX433210	JX433619	JX433828	JX433410	-
New Mexico	*Opsius stactogalus* (Opsiini)	Unknown	Unknown	Kiowa Nat'l Grassland, New Mexico	JX417687	-	JX433620	JX433829	JX433411	-
Fiji	*Opsianus sp.* (Opsiini)	Rutacaea	*Correa* sp.	Viti Levu, Manasavu	JX417691	-	JX433624	JX433833	JX433415	-
Thailand	*Scaphoidella dimidiatus* (Athysanini)	Unknown	Unknown	Khonakaen, Thailand	JX417683	-	-	JX433825	JX433407	-
Zambia	*Mimotettix sp.* (Unplaced)	Unknown	Unknown	Nkuny, Zambia	JX417690	JX433212	JX433623	JX433832	JX433414	-
Taiwan	*Scaphoideus sp.* (Unplaced)	Unknown	Unknown	Nantou, Reiyen Res., Taiwan	JX417685	JX433209	JX433618	JX433827	JX433409	-
Panama	*Osbornellus compressus* (Unplaced)	Unknown	Unknown	Rio Guanche, Panama	JX417684	JX433208	-	JX433826	JX433408	-
Taiwan	*Phlagotettix Cyclops* (Unplaced)	Unknown	Unknown	Taipei, Fushan, Taiwan	JX417688	JX433211	JX433621	JX433830	JX433412	-
Thailand	*Lampridius sp.* (Opsiini)	Unknown	Unknown	Chian Mai, Thailand	JX417689	-	JX433622	JX433831	JX433413	-
Australia	*Orosius orientalis* (Opsiini)	Unknown	Unknown	New So. Wales, unspecified	JX417706	JX433227	JX433639	JX433849	JX433430	JX434025
Australia	*Orosius orientalis* (Opsiini)	Unknown	Unknown	New So. Wales, Mt. Kosciuszko	JX417707	-	-	JX433850	JX433431	JX434026
Australia	*Orosius sp.* (Opsiini)	Amaranthaceae	*Atriplex* sp.	New So. Wales, Founder Gap	JX417691	JX433213	JX433625	JX433834	JX433416	JX434012
***Nesophrosyne* (Opsiini)**										

Hawai`i Island	*"N. sp.125"*	Myrsinaceae	*Myrsine lessertiana*	Mauna Loa, Kau	JX417647	JX433181	JX433582	JX433790	JX433372	JX433981
	"N. sp.253"	Rutaceae	*Melicope volcanicola*	Mauna Loa, South Kona	JX417625	JX433163	JX433559	JX433767	JX433349	JX433961
	"N. sp.254"	Rubiaceae	*Hedyotis terminalis*	Mauna Loa, Stainback Highway	JX417626	JX433164	JX433560	JX433768	JX433350	JX433962
	"N. sp.255"	Campanulaceae	*Clermontia sp.*	Puu Oo Trail, Kahualea Natural Area Reserve	JX417627	JX433165	JX433561	JX433769	JX433351	JX433963
	"N. sp.257"	Campanulaceae	*Clermontia clermontioides*	Mauna Loa, Kau	JX417657	JX433189	JX433592	JX433800	JX433382	JX433990
	"N. sp.259"	Rubiaceae	*Psychotria sp.*	Mauna Loa, Saddle Rd.	-	JX433167	JX433563	JX433771	JX433353	JX433965
	"N. sp.261"	Rubiaceae	*Coprosma rhynchocapra*	Kohala Mtns., Pu'u o Umi	JX417630	JX433169	JX433565	JX433773	JX433355	JX433966
	"N. sp.262"	Urticaceae	*Pipturus albidus*	Kohala Mtns., Pu'u o Umi	JX417631	JX433170	JX433566	JX433774	JX433356	JX433967
	"N. sp.263"	Rutaceae	*Melicope volcanicola*	Mauna Loa, South Kona	JX417632	JX433171	JX433567	JX433775	JX433357	JX433968
	"N. sp.265"	Hydrangeaceae	*Broussaisia arguta*	Mauna Loa, South Kona	JX417633	-	JX433568	JX433776	JX433358	JX433969
	"N. sp.266"	Araliaceae	*Cheirodendron sp.*	Mauna Loa, South Kona	JX417634	-	JX433569	JX433777	JX433359	JX433970
	"N. sp.268"	Scrophulariaceae	*Myoporum sandwicense*	Mauna Loa, Manuka South Kona	JX417635	-	JX433570	JX433778	JX433360	JX433971
	"N. sp.270"	Rubiaceae	*Coprosma sp.*	Mauna Kea, Laupahoehoe	JX417636	JX433172	JX433571	JX433779	JX433361	JX433972
	"N. sp.271"	Urticaceae	*Pipturus albidus*	Mauna Kea, Laupahoehoe	JX417637	JX433173	JX433572	JX433780	JX433362	JX433973
	"N. sp.275"	Amaranthaceae	*Charpentiera sp.*	Mauna Loa, Kipuka Puaulu	JX417639	JX433175	JX433574	JX433782	JX433364	JX433974
	"N. sp.277"	Rubiaceae	*Coprosma rhynchocapra*	Mauna Loa, Koloko Dr., South Kona,	JX417641	-	JX433576	JX433784	JX433366	JX433976
	"N. sp.278"	Rubiaceae	*Coprosma rhynchocapra*	Mauna Loa, South Kona	JX417642	JX433177	JX433577	JX433785	JX433367	JX433977
	"N. sp.280"	Rubiaceae	*Coprosma sp.*	Mauna Loa, Stainback Highway	JX417643	JX433178	JX433578	JX433786	JX433368	JX433978
	"N. sp.283"	Myrsinaceae	*Myrsine lessertiana*	Mauna Kea, Kalopa Forest	JX417644	JX433179	JX433579	JX433787	JX433369	-
	"N. sp.284"	Rubiaceae	*Psychotria hawaiiensis*	Mauna Kea, Kalopa Forest	JX417645	JX433180	JX433580	JX433788	JX433370	JX433979
	"N. sp.287"	Aquifoliaceae	*Ilex anomala*	Mauna Loa, Kau	JX417646	-	JX433581	JX433789	JX433371	JX433980
	"N. sp.289"	Hydrangeaceae	*Broussaisia arguta*	Mauna Loa, Kau	JX417648	-	JX433583	JX433791	JX433373	JX433982
	"N. sp.293"	Aquifoliaceae	*Ilex anomala*	Kohala Mtns, Kohala Mtn. Reserve Tr.	JX417649	JX433182	JX433584	JX433792	JX433374	JX433983
	"N. sp.294"	Campanulaceae	*Clermontia kohalalae*	Kohala Mtns., Kohala Mtn. Reserve Tr.	JX417650	JX433183	JX433585	JX433793	JX433375	JX433984
	"N. sp.295"	Hydrangeaceae	*Broussaisia arguta*	Mauna Loa, Stainback Highway	JX417651	JX433184	JX433586	JX433794	JX433376	JX433985
	"N. sp.297"	Campanulaceae	*Clermontia sp.*	Thurston Lava Tube,	JX417698	JX433219	JX433631	JX433840	JX433425	JX434018

			Hawai`i Volcanoes National Park						
"N. sp.296"	Sapindaceae	*Dodonaea viscosa*	Mauna Loa, Manuka	JX417652	JX433185	JX433587	JX433795	JX433377	JX433986
"N. sp.299"	Rubiaceae	*Coprosma rhynchocapra*	Mauna Loa, Kipuka Puaulu	JX417653	JX433186	JX433588	JX433796	JX433378	JX433987
"N. sp.302"	Rubiaceae	*Coprosma rhynchocapra*	Mauna Loa, Kipuka Puaulu	JX417655	JX433187	JX433590	JX433798	JX433380	JX433989
"N. sp.303"	Campanulaceae	Cyanea stictophylla	Mauna Loa, Stainback Highway	JX417656	JX433188	JX433591	JX433799	JX433381	-
"N. sp.305"	Rutaceae	*Melicope hawaiiensis*	Mauna Loa, Manuka	JX417658	JX433190	JX433593	JX433801	JX433383	JX433991
"N. sp.306"	Rubiaceae	*Coprosma sp.*	Mauna Kea, Pohakuloa Training Area	JX417659	JX433191	JX433594	JX433802	JX433384	JX433992
"N. sp.309"	Rubiaceae	*Psychotria hawaiiensis*	Mauna Loa, Koloko Dr., South Kona	JX417660	JX433192	JX433595	JX433803	JX433385	JX433993
"N. sp.310"	Myrtaceae	*Metrosideros polymorpha*	Mauna Loa, Manuka	JX417661	JX433193	JX433596	JX433804	JX433386	JX433994
"N. sp.360"	Urticaceae	*Urera glabra*	Mauna Loa, Manuka	JX417680	-	JX433615	JX433823	JX433404	-
N. aakokohaikea	Hydrangeaceae	*Broussaisia arguta*	Mauna Loa, Kau - Olaa Forest	JX417552	JX433108	JX433486	JX433695	JX433281	JX433897
N. coma	Rubiaceae	*Psychotria sp.*	Mauna Loa, Kipuka Puaulu	JX417623	JX433162	JX433557	JX433765	JX433347	JX433960
N. eburneola	Rubiaceae	*Psychotria sp.*	Mauna Loa, Manuka	JX417528	JX433091	JX433463	JX433671	JX433257	-
N. giffardi	Scrophulariaceae	*Myoporum sandwicense*	Mauna Loa, Kau	JX417498	JX433065	JX433433	JX433641	JX433229	JX433852
N. giffardi interrupta	Scrophulariaceae	*Myoporum sandwicense*	Mauna Loa, Kipuka Puaulu	JX417499	JX433066	JX433434	JX433642	JX433230	JX433853
N. ignigena	Pittosporaceae	*Pittosporum hosmeri*	Mauna Loa, Manuka	JX417529	-	JX433464	JX433672	JX433258	JX433878
N. insularis	Rubiaceae	*Psychotria hawaiiensis*	Mauna Loa, Kipuka Puaulu	JX417622	JX433161	JX433556	JX433764	JX433346	JX433959
N. kanawao	Hydrangeaceae	*Broussaisia arguta*	Kohala Mtns., Pu'u o Umi	JX417551	-	JX433485	JX433694	JX433280	JX433896
N. mabae	Ebenaceae	*Diospyros sandwicensis*	Road Between Waimea and Kona	JX417527	JX433090	JX433462	JX433670	JX433256	JX433877
N. marginalis	Rubiaceae	*Psychotria hawaiiensis*	Mauna Loa, Kipuka Puaulu	JX417543	JX433104	JX433477	JX433686	JX433272	-
N. montium	Urticaceae	*Pipturus albidus*	Mauna Loa, Kipuka Puaulu - South Kona	JX417629	JX433168	JX433564	JX433772	JX433354	-
N. montivaga	Urticaceae	*Pipturus albidus*	Mauna Loa, Kipuka Ki	JX417654	-	JX433589	JX433797	JX433379	JX433988
N. myrsines	Myrsinaceae	*Myrsine lessertiana*	Mauna Kea, Hamakua Coast,	JX417638	JX433174	JX433573	JX433781	JX433363	-
N. nuenue	Sapindaceae	*Dodonaea viscosa*	Muana Ulu, HAVO	JX417640	JX433176	JX433575	JX433783	JX433365	JX433975
N. oceanides	Rubiaceae	*Psychotria Hawai`iensis*	Mauna Kea, Hamakua Coast,	JX417699	JX433220	JX433632	JX433841	JX433423	-
N. pele	Rutaceae	*Melicope hawaiiensis & M. radiata*	Mauna Loa, Kipuka Puaulu	JX417624	-	JX433558	JX433766	JX433348	-

	N. pluvialis	Rubiaceae	*Coprosma rhynchocapra*	Mauna Loa, Kau	JX417628	JX433166	JX433562	JX433770	JX433352	JX433964
	N. silvicola	Rubiaceae	*Psychotria hawaiiensis hill.*	Mauna Loa, Kona - Kau	JX417497	JX433064	JX433432	JX433640	JX433228	JX433851
Kaua`i	*"N. sp.126"*	Scrophulariaceae	*Myoporum sandwicense*	Polihile Beach	JX417555	JX433111	JX433489	JX433698	JX433284	JX433900
	"N. sp.153"	Unknown	Unknown	Pihea Trail	JX417572	JX433124	JX433506	JX433715	JX433300	JX433912
	"N. sp.154"	Unknown	Unknown	Pihea Trail	JX417573	JX433125	JX433507	JX433716	JX433301	JX433913
	"N. sp.161"	Goodeniaceae	*Scaevola sp.*	Awaawapuhi Trail	JX417574	JX433126	JX433508	JX433717	JX433302	JX433914
	"N. sp.162"	Goodeniaceae	*Scaevola sp.*	Awaawapuhi Trail	JX417576	JX433128	JX433510	JX433719	JX433304	JX433916
	"N. sp.169"	Goodeniaceae	*Scaevola sp.*	Alakai Swamp Tr.	JX417579	JX433129	JX433513	JX433722	JX433307	JX433919
	"N. sp.168"	Araliaceae	*Tetraplasandra sp.*	Kalalau Lookout Rd.	JX417578	-	JX433512	JX433721	JX433306	JX433918
	"N. sp.17"	Urticaceae	*Pipturus sp.*	Canyon Tr.	JX417501	-	JX433436	JX433644	JX433232	JX433854
	"N. sp.120"	Urticaceae	*Pipturus sp.*	Nualolo Cliff Tr.	JX417554	JX433110	JX433488	JX433697	JX433283	JX433899
	"N. sp.170"	Thymelaeaceae	*Wikstroemia sp.*	Alakai Swamp Tr.	JX417580	-	JX433514	JX433723	JX433308	JX433920
	"N. sp.171"	Amaranthaceae	*Charpentiera sp.*	Puu ka Pele Forest	JX417581	JX433130	JX433515	JX433724	JX433309	JX433921
	"N. sp.173"	Araliaceae	*Cheirodendron sp.*	Awaawapuhi Tr.	JX417582	JX433131	JX433516	JX433725	JX433310	JX433922
	"N. sp.174"	Urticaceae	*Urera glabra*	Kalalau Lookout Rd.	JX417583	JX433132	JX433517	-	JX433311	-
	"N. sp.175"	Euphorbiaceae	*Euphorbia celestroides*	Awaawapuhi Tr.	JX417584	JX433133	JX433518	JX433726	-	JX433923
	"N. sp.176"	Rubiaceae	*Psychotria sp.*	Awaawapuhi Tr.	JX417585	JX433134	JX433519	JX433727	JX433312	JX433924
	"N. sp.178"	Rubiaceae	*Psychotria sp.*	Pihea Tr.	JX417586	JX433135	JX433520	JX433728	JX433313	JX433925
	"N. sp.181"	Rubiaceae	*Psychotria sp.*	Awaawapuhi Tr.	JX417575	JX433127	JX433509	JX433718	JX433303	JX433915
	"N. sp.182"	Urticaceae	*Boehmeria grandis*	Nualolo Cliff Tr.	JX417587	JX433136	JX433521	JX433729	JX433314	-
	"N. sp.184"	Araliaceae	*Tetraplasandra sp.*	Blue Hole	JX417588	-	JX433522	JX433730	JX433315	JX433926
	"N. sp.185"	Urticaceae	*Urera glabra*	Wainiha Valley	JX417589	JX433137	JX433523	JX433731	JX433316	JX433927
	"N. sp.21"	Goodeniaceae	*Scaevola procera*	Kalalau Lookout Rd.	JX417503	JX433069	JX433438	JX433646	JX433234	-
	"N. sp.218"	Rubiaceae	*Psychotria sp.*	Kalalau Lookout Rd., Puu O Kila, Kokee STP	JX417605	-	JX433539	JX433747	JX433332	JX433943
	"N. sp.23"	Campanulaceae	*Clermontia fauriei*	Alakai Swamp Tr.	JX417516	JX433080	JX433451	JX433659	JX433246	JX433866
	"N. sp.25"	Euphorbiaceae	Syzigium sandwicensis	Nualolo Tr.	JX417504	JX433070	JX433439	JX433647	-	JX433856
	"N. sp.26"	Rubiaceae	*Psychotria sp. and Hedyotis sp.*	Puu ka Pele Forest	JX417506	JX433072	JX433441	JX433649	JX433236	JX433857
	"N. sp.29"	Sapindaceae	*Dodonaea viscosa*	Kohua Tr.	JX417577	-	JX433511	JX433720	JX433305	JX433917
	"N. sp.353"	Unknown	Unknown	Kawakoi Stream	JX417675	JX433204	JX433610	JX433818	JX433400	JX434007
	"N. sp.54"	Goodeniaceae	*Scaevola sp.*	Kalalau Lookout Rd.	JX417517	JX433081	JX433452	JX433660	JX433247	JX433867
	N. heopoko	Hydrangeaceae	*Broussaisia arguta*	Alakai Swamp Tr.	JX417502	JX433068	JX433437	JX433645	JX433233	JX433855
	N. umbratilis	Rubiaceae	*Psychotria and Hedyotis*	Kuia Napali-Kona Forest Reserve	JX417505	JX433071	JX433440	JX433648	JX433235	-
Lana`i	*"N. sp.15"*	Rubiaceae	*Psychotria mariana*	Munro Tr.	JX417500	JX433067	JX433435	JX433643	JX433231	-
	"N. sp.244"	Myrtaceae	*Metrosideros polymorpha*	Munro Tr.	JX417620	JX433160	JX433554	JX433762	-	JX433957
	"N. sp.245"	Goodeniaceae	*Scaevola sp.*	Munro Tr.	JX417621	-	JX433555	JX433763	JX433345	JX433958
	N. caelicola	Rubiaceae	*Psychotria sp.*	Munro Tr.	JX417619	JX433159	JX433553	JX433761	JX433344	JX433956

	N. lineata	Rubiaceae	*Hedyotis terminalis*	Munro Tr.	JX417510	-	JX433445	JX433653	JX433240	JX433861
Maui	*"N. sp.55"*	Sapindaceae	*Dodonaea viscosa*	Haleakala Mtn., Kaupo Gap	JX417518	JX433082	JX433453	JX433661	JX433248	JX433868
	"N. sp.101"	Rubiaceae	*Coprosma sp.*	Mauna Kahalawai, Puu Kukui	JX417546	JX433106	JX433480	JX433689	JX433275	JX433891
	"N. sp.102"	Sapindaceae	*Dodonaea viscosa*	Mauna Kahalawai, Puu kukui	JX417547	-	JX433481	JX433690	JX433276	JX433892
	"N. sp.104"	Rubiaceae	*Psychotria sp.*	Mauna Kahalawai, Puu kukui	JX417548	-	JX433482	JX433691	JX433277	JX433893
	"N. sp.105"	Rubiaceae	*Psychotria mariana*	Mauna Kahalawai, Waihee Tr.	JX417549	JX433107	JX433483	JX433692	JX433278	JX433894
	"N. sp.117"	Araliaceae	*Cheirodendron trigynum*	Haleakala Mtn., Kaupo Gap	JX417553	JX433109	JX433487	JX433696	JX433282	JX433898
	"N. sp.127"	Sapindaceae	*Dodonaea viscosa*	Haleakala Mtn., HWY 31 stop	JX417556	-	JX433490	JX433699	JX433285	-
	"N. sp.130"	Rutaceae	*Melicope sp.*	Haleakala Mtn., Kaupo Gap Tr.	JX417558	JX433113	JX433492	JX433701	JX433287	-
	"N. sp.131"	Unknown	gen. sweep	Haleakala Mtn., Kaupo Gap	JX417559	-	JX433493	JX433702	JX433288	JX433901
	"N. sp.132	Blechnaceae	*Sadleria* fern	Haleakala Mtn., Kaupo Gap	JX417560	JX433114	JX433494	JX433703	JX433289	JX433902
	"N. sp.137"	Gesneriaceae	*Cyrtandra platyphylla*	Haleakala Mtn., Makawao Forest	JX417561	JX433115	JX433495	JX433704	JX433290	-
	"N. sp.138"	Unknown	Unknown	Haleakala Mtn., Makawa Forest	JX417562	-	JX433496	JX433705	JX433291	JX433903
	"N. sp.139"	Rubiaceae	*Coprosma sp.*	Haleakala Mtn., Upper Waikamoi	JX417563	-	JX433497	JX433706	-	JX433904
	"N. sp.143"	Urticaceae	*Pipturus albidus*	Haleakala Mtn., Makawa Forest	JX417565	JX433117	JX433499	JX433708	JX433293	JX433906
	"N. sp.144"	Rubiaceae	*Coprosma sp.*	Haleakala Mtn., Makawa Forest	JX417566	JX433118	JX433500	JX433709	JX433294	JX433907
	"N. sp.146"	Goodeniaceae	*Scaevola sp.*	Haleakala Mtn., Makawa Forest	JX417567	JX433119	JX433501	JX433710	JX433295	JX433908
	"N. sp.148"	Rubiaceae	*Coprosma montana*	Haleakala Mtn., Makawa Forest	JX417569	JX433121	JX433503	JX433712	JX433297	JX433910
	"N. sp.150"	Unknown	Unknown	Haleakala Mtn., Haiku Uka	JX417571	JX433123	JX433505	JX433714	JX433299	JX433911
	"N. sp.312"	Rutaceae	*Melicope sp.*	Mauna Kahalawai, Puu kukui	JX417662	JX433194	JX433597	JX433805	JX433387	JX433995
	"N. sp.316"	Rutaceae	Psychotria sp.	Mauna Kahalawai, Puu kukui	JX417663	JX433195	JX433598	JX433806	JX433388	JX433996
	"N. sp.317"	Unknown	gen. sweep	Mauna Kahalawai, Puu kukui	JX417664	JX433196	JX433599	JX433807	JX433389	JX433997
	"N. sp.318"	Urticaceae	*Pipturus albidus*	Mauna Kahalawai, Puu kukui	JX417665	JX433197	JX433600	JX433808	JX433390	JX433998

"N. sp.319"	Unknown	Unknown	Mauna Kahalawai, Puu kukui	JX417666	JX433198	JX433601	JX433809	JX433391	JX433999
"N. sp.321"	Rutaceae	*Melicope clusiifolia*	Mauna Kahalawai, Puu kukui	JX417667	-	JX433602	JX433810	JX433392	JX434000
"N. sp.322"	Unknown	Unknown	Mauna Kahalawai, Puu kukui	JX417668	JX433199	JX433603	JX433811	JX433393	JX434001
"N. sp.357"	Hydrangeaceae	*Broussaisia arguta*	Mauna Kahalawai, Pohakea Gulch	JX417677	-	JX433612	JX433820	JX433402	JX434009
"N. sp.358"	Unknown	*Lobelia gloria-montis*	Mauna Kahalawai, Pohakea Gulch	JX417678	JX433205	JX433613	JX433821	JX433403	JX434010
"N. sp.359"	Urticaceae	*Urera glabra*	Mauna Kahalawai, Ae Stream	JX417679	-	JX433614	JX433822	-	JX434011
"N. sp.44"	Dipentodontaceae	*Perrottetia sp.*	Haleakala Mtn., Makawao Forest	JX417512	JX433077	JX433447	JX433655	JX433242	JX433863
"N. sp.48"	Urticaceae	*Pipturus albidus*	Haleakala Mtn., Kaupo Gap Tr.	JX417514	JX433079	JX433449	JX433657	JX433244	JX433864
"N. sp.57"	Rubiaceae	*Coprosma foliosa*	Haleakala Mtn., Kaupo Gap Tr.	JX417564	JX433116	JX433498	JX433707	JX433292	JX433905
"N. sp.58"	Rubiaceae	*Coprosma sp.*	Haleakala Mtn., Lower Makawao Forest	JX417519	JX433083	JX433454	JX433662	JX433249	JX433869
"N. sp.65"	Rutaceae	*Melicope clussifolia*	Haleakala Mtn., Lower Makawao Forest	JX417522	JX433086	JX433457	JX433665	JX433252	JX433872
"N. sp.66"	Rutaceae	*Melicope clussifolia*	Haleakala Mtn., Lower Makawao Forest	JX417523	-	JX433458	JX433666	JX433253	JX433873
"N. sp.67"	Campanulaceae	*Clermontia arborescence*	Haleakala Mtn., Lower Makawao	JX417524	JX433087	JX433459	JX433667	JX433254	JX433874
"N. sp.69"	Rubiaceae	*Coprosma sp.*	Mauna Kahalawai, Puu Kukui	JX417525	JX433088	JX433460	JX433668	JX433255	JX433875
"N. sp.99"	Rubiaceae	*Hedyotis terminalis*	Mauna Kahalawai, Puu kukui	JX417544	-	JX433478	JX433687	JX433273	-
N. anguilifera	Rubiaceae	*Coprosma foliosa*	Haleakala Mtn., Makawao Forest - Kaupo Gap	JX417520	JX433084	JX433455	JX433663	JX433250	JX433870
N. broussaisiai	Hydrangeaceae	*Broussaisia arguta*	Mauna Kahalawai, Puu kukui	JX417545	JX433105	JX433479	JX433688	JX433274	JX433890
N. caelicola	Rubiaceae	*Psychotris sp.*	Mauna Kahalawai, Waihee Tr.	JX417619	JX433159	JX433553	JX433761	JX433344	JX433956
N. cinera	Rubiaceae	*Coprosma montana*	Haleakala Mtn., Lower Makawao Forest	JX417568	JX433120	JX433502	JX433711	JX433296	JX433909
N. furculata	Campanulaceae and Rubicaceae	*Lobelia sp. and Coprosma*	Haleakala Mtn., Makawao Forest - Kaupo Gap	JX417521	JX433085	JX433456	JX433664	JX433251	JX433871
N. haleakala	Rubiaceae	*Coprosma montana*	Haleakala Mtn., Silver Sword Loop Tr.	JX417515	-	JX433450	JX433658	JX433245	JX433865
N. imbricola	Myrsinaceae	*Myrsine lessertiana*	Haleakala Mtn., Kaupo	JX417513	JX433078	JX433448	JX433656	JX433243	-

				Gap Tr.						
	N. kaupoi	Hydrangeaceae	*Broussaisia arguta*	Haleakala Mtn., Kaupo Gap	JX417550	-	JX433484	JX433693	JX433279	JX433895
	N. nimbicola	Myrsinaceae	*Myrsine lessertiana*	Haleakala Mtn., Kaupo Gap Tr.	JX417557	JX433112	JX433491	JX433700	JX433286	-
	N. obliqua	Rubiaceae	*Coprosma montana*	Mauna Kahalawai, Pohakea Gulch	JX417676	-	JX433611	JX433819	JX433401	JX434008
	N. ogradyi	Hydrangeaceae	*Broussaisia arguta*	Haleakala Mtn., Makawao Forest	JX417511	JX433076	JX433446	JX433654	JX433241	JX433862
	N. oneanea	Rubiaceae	*Coprosma sp. (big leaf)*	Haleakala Mtn., Makawao Forest	JX417570	JX433122	JX433504	JX433713	JX433298	-
Moloka`i	*"N. sp.15"*	Rubiaceae	*Psychotria mariana*	Kamakou TNC, Boardwalk Tr.	JX417500	JX433067	JX433435	JX433643	JX433231	-
	"N. sp.220"	Rubiaceae	*Psychotria sp.*	Hanalilolilo Look Out	JX417607	-	JX433541	JX433749	JX433334	JX433945
	"N. sp.221"	Unknown	gen. sweep	Puu Kolekole, Dry South-Side	JX417608	JX433152	JX433542	JX433750	-	JX433946
	"N. sp.233"	Sapindaceae	*Dodonaea viscosa*	Kumwela Rd. Kamakou TNC Preserve	JX417697	JX433218	JX433630	JX433839	JX433421	JX434017
	"N. sp.225"	Rubiaceae	*Coprosma foliosa*	Kamakou TNC Preserve, Cabin	JX417609	-	JX433543	JX433751	JX433335	JX433947
	"N. sp.227"	Urticaceae	*Urera glabra*	Kamakou, Puu Kolekole	JX417610	JX433153	JX433544	JX433752	-	JX433948
	"N. sp.228"	Unknown	Unknown	Kamakou TNC, Boardwalk Tr.	JX417611	JX433154	JX433545	JX433753	JX433336	JX433949
	"N. sp.236"	Unknown	Unknown	Kumwela Rd., Kamakou TNC Preserve	JX417615	JX433156	JX433549	JX433757	JX433340	JX433952
	"N. sp.237"	Rubiaceae	*Coprosma foliosa*	Kamakou TNC Preserve, Cabin	JX417616	-	JX433550	JX433758	JX433341	JX433953
	"N. sp.241"	Rubiaceae	*Psychotria sp.*	Kamakou TNC, Boardwalk Tr.	JX417617	JX433157	JX433551	JX433759	JX433342	JX433954
	"N. sp.242"	Scrophulariaceae	*Myoporum sandwicense*	Kumwela Rd., Kamakou TNC Preserve	JX417618	JX433158	JX433552	JX433760	JX433343	JX433955
	"N. sp.229"	Unknown	Unknown	Puu Kolekole, Kamakou TNC Preserve	JX417612	-	JX433546	JX433754	JX433337	JX433950
	N. magnaccai	Hydrangeaceae	*Broussaisia arguta*	Kamakou, Puu Kolekole	JX417526	JX433089	JX433461	JX433669	-	JX433876
	N. milu	Myrsinaceae	*Myrsine lessertiana*	Kamakou, Puu Kolekole	JX417614	-	JX433548	JX433756	JX433339	JX433951
	N. obliqua	Rubiaceae	*Coprosma sp.*	Kamakou, Puu Kolekole	JX417676	-	JX433611	JX433819	JX433401	JX434008
	N. paludicola	Unknown	gen. sweep	Kamakou, Puu Kolekole	JX417613	JX433155	JX433547	JX433755	JX433338	-
	N. procellaris	Thymelaeaceae	*Wikstroemia sp.*	Puu Kolekole, Mauna Kahalawai	JX417693	JX433214	JX433626	JX433835	JX433417	JX434013
O`ahu	*"N. sp.189"*	Unknown	gen. sweep	Koolau Mtns. WiliWiliNui Trail	JX417591	JX433139	JX433525	JX433733	JX433318	JX433929
	"N. sp.201"	Rubiaceae	*Hedyotis terminalis*	Mt. Kaala, Pahole Ridge Tr.	JX417594	-	JX433528	JX433736	JX433321	JX433932
	"N. sp.202"	Urticaceae	*Urera glabra*	Waianae Mtns, Kaluaa Gulch	JX417595	JX433142	JX433529	JX433737	JX433322	JX433933

"N. sp.208"	Rubiaceae	Morinda trimera	Waianea Mtns, Kaluaa Gulch	JX417597	JX433144	JX433531	JX433739	JX433324	JX433935
"N. sp.210"	Unknown	Unknown	Koolau Mtns., Hawai`i Loa Ridge	JX417598	-	JX433532	JX433740	JX433325	JX433936
"N. sp.213"	Euphorbiaceae	*Cloaxylon sandwicense*	Waianea Mtns, Puu Hapapa Shelf	JX417601	JX433147	JX433535	JX433743	JX433328	JX433939
"N. sp.214"	Amaranthaceae	*Charpentiera sp.*	Koolau Mtns., Kului Gulch	JX417602	JX433148	JX433536	JX433744	JX433329	JX433940
"N. sp.215"	Rubiaceae	*Psychotria sp.*	Waianea Mtns, Puu Hapapa Shelf	JX417603	JX433149	JX433537	JX433745	JX433330	JX433941
"N. sp.216"	Urticaceae	*Urera glabra*	Waianae Mtns., Palikea	JX417604	JX433150	JX433538	JX433746	JX433331	JX433942
"N. sp.33"	Aquifoliaceae	*Ilex anomala*	Mt. Kaala, Summit Boardwalk	JX417507	JX433073	JX433442	JX433650	JX433237	JX433858
"N. sp.246"	Scrophulariaceae	*Myoporum sandwicense*	Waianae Mtns., Kaena Point	JX417702	-	JX433635	JX433844	JX433426	JX434022
"N. sp.341"	Rubiaceae	*Psychotria sp.*	Koolau Mtns., Poamoho Tr.	JX417703	JX433217	JX433629	JX433838	JX433420	JX434016
"N. sp.343"	Hydrangeaceae	*Broussaisia arguta*	Koolau Mtns., Poamoho Tr.	JX417670	-	JX433605	JX433813	JX433395	JX434003
"N. sp.344"	Thymelaeaceae	*Wikstroemia sp.*	Koolau Mtns., Poamoho Tr.	JX417671	JX433200	JX433606	JX433814	JX433396	JX434004
"N. sp.345"	Myrtaceae	*Metrosideros polymorpha*	Koolau Mtns., Poamoho Tr.	JX417672	JX433201	JX433607	JX433815	JX433397	JX434005
"N. sp.347"	Oleaceae	*Nestegis sp.*	Koolau Mtns., Poamoho Tr.	JX417674	JX433203	JX433609	JX433817	JX433399	JX434006
N. oreadis	Thymelaeaceae	*Wikstroemia oahuensis*	Koolau Mtns., Poamoho Tr.	JX417704	JX433224	JX433637	JX433846	JX433428	-
"N. sp.36"	Myrtaceae	*Metrosideros polymorpha*	Waianae Mtns., Pahole	JX417508	JX433074	JX433443	JX433651	JX433238	JX433859
"N. sp.90"	Goodeniaceae	*Scaevola mollis*	Koolau Mtns., Puaoa Flat Trail	JX417535	JX433097	JX433470	JX433678	JX433264	JX433883
"N. sp.91"	Rubiaceae	*Coprosma foliosa*	Mt. Kaala, Pahole Ridge Tr.	JX417536	JX433098	JX433471	JX433679	JX433265	JX433884
"N. sp.92"	Oleaceae	*Nestegis sandwicensis*	Mt. Kaala, Pahole Ridge Tr.	JX417537	JX433099	JX433472	JX433680	JX433266	JX433885
"N. sp.93"	Oleaceae	*Nestegis sandwicensis*	Mt. Kaala, Pahole Ridge Tr.	JX417538	JX433100	JX433473	JX433681	JX433267	JX433886
"N. sp.94"	Rubiaceae	*Psychotria mariana*	Mt. Kaala, Pahole Ridge Tr.	JX417539	-	JX433474	JX433682	JX433268	JX433887
"N. sp.95"	Rubiaceae	*Psychotria sp.*	Koolau Mtns. Wiliwilinui Trail	JX417540	JX433101	JX433475	JX433683	JX433269	JX433888
"N. sp.96"	Aquifoliaceae	*Ilex anomala*	Koolau Mtns. Wiliwilinui Trail	JX417541	JX433102	-	JX433684	JX433270	-
"N. sp.97"	Rubiaceae	*Hedyotis terminalis*	Mt. Kaala, Summit Boardwalk	JX417542	JX433103	JX433476	JX433685	JX433271	JX433889

N. bobea	Rubiaceae	*Bobea eliator*	Koolau Mtns. Wiliwilinui Trail	JX417533	JX433095	JX433468	JX433676	JX433262	-
N. makaihe	Hydrangeaceae	*Broussaisia arguta*	Mt. Kaala, Summit Boardwalk	JX417509	JX433075	JX433444	JX433652	JX433239	JX433860
N. maritima	Sapindaceae	*Dodonaea viscosa*	Mt. Kaala, Along Rd. to Summit	JX417694	JX433215	JX433627	JX433836	JX433418	JX434014
N. maritima	Sapindaceae	*Dodonaea viscosa*	Diamond Head Crater	JX417673	JX433202	JX433608	JX433816	JX433398	-
N. monticola	Rubiaceae	*Coprosma ochracera*	Mt. Kaala, Summit Boardwalk.	JX417532	JX433094	JX433467	JX433675	JX433261	JX433880
N. notatula	Myrtaceae	*Metrosideros polymorpha*	Koolau Mtns., Poamoho Tr.	JX417534	JX433096	JX433469	JX433677	JX433263	JX433882
N. palolo	Rutaceae	*Melicope sp.*	Mt. Kaala, Coulvert 59	JX417531	JX433093	JX433466	JX433674	JX433260	JX433879
N. peleae	Rutaceae	*Melicope sp.*	Mt. Kaala, Coulvert 59	JX417590	JX433138	JX433524	JX433732	JX433317	JX433928
N. pipturi	Urticaceae	*Pipturus albidus*	Koolau Mtns., Mt. Tantalus, Manoa Cliff Trail	JX417592	JX433140	JX433526	JX433734	JX433319	JX433930
"N. sp.219"	Urticaceae	*Pipturus albidus*	Mt. Kaala, Pahole Ridge Tr.,	JX417606	JX433151	JX433540	JX433748	JX433333	JX433944
"N. sp.197"	Urticaceae	*Pipturus albidus*	Koolau, Mtns., Kuliouou Gulch,	JX417593	JX433141	JX433527	JX433735	JX433320	JX433931
N. ponapona	Urticaceae	*Pipturus albidus*	Koolau Mtns., Mt. Tantalus Dr.	JX417599	JX433145	JX433533	JX433741	JX433326	JX433937
"N. sp.212"	Urticaceae	*Pipturus albidus*	Waianae Mtns., Pia Valley	JX417600	JX433146	JX433534	JX433742	JX433327	JX433938
N. touchardii	Urticaceae	*Touchardia latifolia*	Koolau Mtns., Pali Lookout	JX417596	JX433143	JX433530	JX433738	JX433323	JX433934
"N. sp.246"	Scrophulariaceae	*Myoporum sandwicense*	Koolau Mtns., Makapuu Point	JX417702	-	JX433635	JX433844	JX433426	JX434022
"N. sp.340"	Scrophulariaceae	*Myoporum sandwicense*	Waianae Mtns., Kaena Point	JX417669	-	JX433604	JX433812	JX433394	JX434002
			Percent Gene Coverage for Taxon Sampling:	99%	78%	98%	99%	96%	84%

CHAPTER 3

Historical biogeography and ecological opportunity in the adaptive radiation of native Hawaiian leafhoppers (Cicadellidae: *Nesophrosyne*)

ABSTRACT

Aim The aim of this study is to infer the roles of ecology and geology in the adaptive radiation, historical biogeography, and species diversification dynamics of the native Hawaiian leafhoppers (Hemiptera: Cicadellidae: *Nesophrosyne*).

Location The Hawaiian Archipelago

Methods A six gene molecular data set for 191 *Nesophrosyne* species was used to reconstruct phylogenetic relationships in absolute time with relaxed Bayesian methods. Dated phylogenetic hypotheses were used to reconstruct ancestral range and host-plant associations for the genus. Phylogenetic clustering methods were implemented to examine the influence of island geography on the inter- and intra-island diversification patterns. Lineage diversification dynamics were inferred using the γ-statistic and birth-death likelihood methods.

Results *Nesophrosyne* split from a common ancestor with its sister genus 4.5 million years ago (Ma), with a basal divergence on Hawai`i 3.2 Ma. Ancestral range reconstructions reveal a Kaua`i origin, with a subsequent progression-rule pattern of island colonization. Ancestral host-plant associations reconstruct Urticaceae and Rubiaceae at the root, with subsequent host transitions resulting in host-specific clades. Kaua`i is the only phylogenetically clustered island; however, species show significant terminal clustering for the four main islands. Diversification rates in *Nesophrosyne* show an initial burst in speciation rates with a diversity-dependent decline.

Main Conclusions *Nesophrosyne* represents a comparatively young group. Results suggest host arrival times and insect-insect competition have been important in diversification patterns. The plant families Urticaceae and Rubiaceae played important roles in the early diversification of *Nesophrosyne*. Continued host transitions have resulted in host-associated clades and permitted inter-island colonization. Island geography have imposed significant barriers to continued gene flow, leading to extensive allopatric speciation and intra-island diversification. A high initial speciation rate was associated with host transitions and the formation of complex island structures. Diversification rates exhibit a diversity-dependent decline, corresponding to island formation. Results construct a model for understanding adaptive radiation in herbivorous insects.

Key Words *Nesophrosyne*, Auchenorrhyncha, Hawai`i, Biogeography, Adaptive Radiation, Ecological Opportunity, Phylogenetic Clustering, Diversity Dynamics

"*One could stand on that mountain [Mauna Loa, Hawai`i], and… could see all the climes of the world at a single glance of the eye."* Mark Twain, *Mark Twain's letters from Hawai`i* (1966).

INTRODUCTION

The existence of species is hypothesized as the natural tendency of life to fill discrete geographic and ecological niches (Dobzhansky, 1951; Coyne & Orr, 2004). This concept is the backbone of such evolutionary theories as adaptive and nonadaptive species radiation. Adaptive radiation describes rapid species diversification to fill novel ecological opportunity, while non-adaptive radiation describes species diversification resulting from allopatric or paraptric isolation (Simpson, 1953; Schluter, 2000; Rundell & Price, 2009; Glor, 2010). Both theories are thought to operate together in large, complex species radiations and help to explain organismal diversity and its distributions at local and global scales (Rundell & Price, 2009; Glor, 2010; Losos, 2010). Both ecological and geographic mechanisms are thought to play important roles in the adaptive diversification of organismal groups (Schluter, 2000; Rundell & Price, 2009; Losos, 2010), but these processes remain poorly understood (Yoder *et al.*, 2010). Disentangling these drivers requires phylogenetically well circumscribed systems coupled with ecological and geographic information (Glor, 2010; Losos, 2010).

Oceanic islands offer a powerful framework to tease apart the drivers of diversification because ecological opportunities are formed *de novo*, their discrete geophysical ranges provide tractable study systems, and extensive theory predicts their controls on endemic diversity (MaCArthur & Wilson, 1967; Whittaker *et al.*, 2008; Gillespie & Baldwin, 2010). These factors are perhaps best understood for the Hawaiian archipelago, where the geologic formation and island ages are well understood. Island formation followed a simple linear geo-chronological progression, but resulted in complex, often multi-volcano islands with replicated habitat structures (Fig. 1: Carson & Clague, 1995). Prior to the ~5 million year age of the current high islands, erosional processes reduced the older North West islands to low-lying atolls, eliminating most endemic lineages (Price & Clague, 2002). This pre-Kaua`i gap resulted in a relatively young endemic biota derived almost entirely by *in situ* speciation, resulting in unprecedented levels of endemism: 99% for insects and 89% for plants (Miller & Eldridge, 1996; Wagner *et al.*, 1999). Biogeographic theory predicts that early colonizing lineages established on the oldest islands tend to follow a "progression rule" pattern of geo-synchronous colonization of younger islands (Wagner & Funk, 1995). Upon colonization, such lineages were presented with novel ecological opportunity, relaxed selection pressures, and a dynamic landscape, which can propel initial bursts in speciation rates and rates of ecological disparity and trait diversification (Gillespie & Baldwin, 2010; Glor, 2010; Mahler *et al.*, 2010; Rabosky & Glor, 2010). As resource limits are reached, diversity-dependent effects should cause speciation rate to decline (Rabosky & Lovette, 2008b; Rabosky & Glor, 2010); however, this pattern remains untested for Hawaiian groups.

Hawai`i is home to some of the most iconic adaptive radiations known, including Hawaiian *Drosophila* (O'Grady & Desalle, 2008), *Tetragnatha* (Gillespie, 2004), and silverswords (Baldwin & Sanderson, 1998). These examples serve as model systems for understanding how macroevolutionary processes shape organismal diversity. Missing from this picture are Hawai`i's poorly known phytophagous insect groups – particularly in the sap-feeding insect suborder Auchenorrhyncha (Hemiptera), which are a major fraction of Hawaii's

disharmonic species diversity (Roderick & Percy, 2008). Phytophagous insects provide a unique opportunity to directly examine the role of ecological opportunity in island radiations because they are obligately associated with the endemic flora and exhibit high levels of host-specificity (Price, 2008; Roderick & Percy, 2008). Host-plants provide fundamental ecological opportunities and complex habitats for adaptive diversification to occur, and are known to increase local insect diversity, geographic ranges, and morphological diversity (Cocroft *et al.*, 2008; Price, 2008). On Hawai`i, this ecological opportunity is expected to directly shape insect diversification in a similar fashion to islands themselves, because plants offer dynamic and diverse niche space, although geological controls, insect-insect competition, and plant physiology will limit host availability (Joy & Crespi, 2012).

This study focuses on one of the largest phytophagous insect groups endemic to Hawai`i, as a model to understand the roles of geographic and ecological opportunity in insect diversification. The native Hawaiian leafhoppers (Cicadellidae: *Nesophrosyne*) provide one of the best-understood endemic herbivore radiations, for which ecological and host-plant associations are well known (Zimmerman, 1948; Bennett & O'Grady, 2012). *Nesophrosyne* comprises at least 190 species, derived from a single western Pacific Rim colonization (Bennett & O'Grady, 2012). Species are highly host-plant specific and ecologically specialized, forming monophyletic associations with endemic plant lineages. Moreover, *Nesophrosyne* exhibits quintessential characteristics of an adaptive radiation, including dramatic morphological adaptations to the endemic Hawaiian flora, and diversification across the archipelago to fill all habitat types from coastal to sub-alpine regions (Zimmerman, 1948; Bennett & O'Grady, 2012).

We address the underlying question of how ecological and geologic opportunities influence species diversification in *Nesophrosyne* by 1) inferring molecular divergence times, 2) reconstructing ancestral ranges and geological constraints on dispersal, 3) inferring ancestral host-plant associations, and 4) examining species diversification dynamics. This study is the first to apply comprehensive comparative phylogenetic methods to tease apart the drivers of adaptive radiation in Hawaiian insect groups and for the suborder Auchenorrhyncha. Our results develop a model understanding of how ecological and geological controls shape adaptive diversification in insects.

MATERIALS AND METHODS

Data

Molecular analyses were conditioned on absolute time, using the multi-gene molecular dataset from Bennett and O'Grady (2012). Taxonomic sampling includes 191 *Nesophrosyne* species in 198 individuals, and two outgroup species from the inferred sister genus, *Orosius* (Bennett and O'Grady, 2012). Genetic data comprises a total of six genes (3520 base pairs) from mitochondrial (*Cytochrome Oxidase I & II,* and 16S), nuclear (*Histone 3* and *Wingless*), and bacterial endosymbiont (16S) genomes. Species range and host-plant data for historical biogeographic and ancestral state reconstruction were taken from previous systematic work on *Nesophrosyne* (Zimmerman, 1948; Bennett & O'Grady, 2011, 2012). The dominant host-plant associations are available for 175 species (approximately 92%), which include plant family and genus-level identifications.

Molecular dating

Molecular dating approaches were conducted using Bayesian methods in BEAST v1.6.2 on XSEDE in CIPRES (Drummond & Rambaut, 2007; Miller *et al.*, 2009). MCMC runs were implemented under a relaxed uncorrelated lognormal model, allowing for simultaneous estimation of phylogeny and divergence times, uncertainty in calibration specification, and rate heterogeneity among branches (Drummond *et al.*, 2006). Molecular data were fully partitioned by codon, determined previously (Bennett & O'Grady, 2012).

Calibrating molecular phylogenies in absolute time is non-trivial, requiring prior knowledge derived from the fossil record or geologic information (Drummond *et al.*, 2006). No fossil calibrations are available for *Nesophrosyne* or the leafhopper subfamily Deltocephalinae to calibrate phylogenetic inference (Zahniser & Dietrich, 2010). However, the Hawaiian Islands offer a unique and well-tested suite of geologic calibrations to infer the absolute ages of endemic groups (Carson & Clague, 1995; Fleischer *et al.*, 1998). Fleischer et al. (1998) proposed a set of criteria for determining geologic calibrations, which were addressed objectively by reconstructing ancestral geographic ranges on the Bayesian phylogeny from Bennett and O'Grady (2012) with maximum likelihood in MESQUITE v2.75 (results not shown: Maddison & Maddison, 2011). Calibrations were selected for lineages exhibiting highly supported progression-rule pattern at highly supported nodes (PP > 95). A total of nine calibration points were identified (see Fig. 2), corresponding to Kaua`i, Maui, and Hawai`i Island. An emphasis was placed on selected two-island calibration strategy, targeting reciprocally monophyletic sister radiations on Maui and Hawai`i Island to reduce assumptions about basal divergence times. The crown node for Clade A was selected for an older calibration point since all basally diverging nodes reconstruct as Kaua`i, and it represents the most basally supported node other than the root. Normally distributed probabilistic priors with means centered on the biologically relevant age of each island were set as follows (Carson & Clague, 1995): Hawai`i Island, Mean = 0.5 Ma (95% range = 0.25 – 0.75 Ma); Maui, mean = 1.3 Ma (95% range 1.05 – 1.55 Ma); and, Kaua`i, mean = 5.0 Ma (95% range = 4.17 – 5.82 Ma). The 95% intervals were set to allow for uncertainty in island colonization times, and was increased for Kaua`i reflecting the long geologic period during which it existed as the only high island (3.7 – 5.1 Ma; Carson & Clague, 1995).

BEAST analyses were run four times for 1×10^9 generations, and sampled every 4000^{th} iteration. Searches were initiated from a randomly generated starting tree under a Yule prior. Preliminary analyses were run to explore the effect of prior choice by excluding the older Kaua`i calibration and narrowing the standard deviations of younger calibrations from .15 to .1, which had marginal influence on posterior estimates (data not shown). Convergence and burn-in was assessed using the Estimated Sample Size (ESS > 200) in TRACER v1.5 and cumulative posterior probability plots AWTY (Rambaut & Drummond, 2007; Nylander *et al.*, 2008). Convergence diagnostics indicated a 25% burn-in was adequate to remove preceding tail of MCMC iterations. A maximum clade credibility tree (MCCT) was summarized for 5,000 resampled post burn-in trees combined from each run (20,000 trees total).

Historical biogeographic and ancestral host-plant reconstructions

The historical biogeography of *Nesophrosyne* was reconstructed using Dispersal-Extinction-Cladogensis (DEC) maximum likelihood ancestral range reconstruction method in

LAGRANGE v.2 (Ree & Smith, 2008). LAGRANGE was chosen for its incorporation of uncertainty in ancestral range reconstructions, modeling of paleogeographic information, inclusion of dispersal and extinction, and increased accuracy over other methods, which is desirable for island systems (Ree & Smith, 2008; Buerki *et al.*, 2011).

Ancestral range areas were set as a seven-state model, corresponding to the individual major islands of the Hawaiian Archipelago: Kaua`i (K), O`ahu (O), Moloka`i (Mo), Lana`i (L), Maui (M), and Hawai`i Island (H). The ancestral range for *Orosius* was coded as Australia (Au). Reconstructions were conditioned on the MCCT tree. Four ancestral range models were run: i) unconstrained (UM-U), ii) unconstrained two-area ancestral state (UM-2), and ii) time-stratified unconstrained (ST-U), and iv) a time-stratified two-area ancestral state (ST-2). The stratified models imposed a series of rate matrices that disallowed range inheritance between islands prior to their emergence (depicted in Fig. 1: Carson & Clague, 1995). We tested a restricted two-state historical range model, because the DEC requires all terminal states be considered in the inference of ancestral nodes, leading to inflated uncertainty (Ree & Sanmartín, 2009; Buerki *et al.*, 2011). All models allowed equal transition rates between islands, reflecting the lack of information regarding inter-island dispersal rates. Ancestral range model performance was evaluated based on geological plausibility (*e.g.,* inferred ancestral ranges v. island formation times) and with a statistically significant confidence window of >2 log-likelihood (Ree & Smith, 2008).

Ancestral host-plant associations for *Nesophrosyne* species were reconstructed with maximum likelihood criteria in MESQUITE v2.75 (Maddison & Maddison, 2011). Reconstructions were performed on the MCCT, under a Markov *K*-state 1 (MK1) model of equally probable transition rates. Host-plant family categorical data (22 states) was selected over the genus level for computational feasibility. A simpler model was implemented here, because the number of possible transition rates for ancestral host-plant associations is large. Host-plants were coded as one of twenty-two states according to host-plant family, following Bennett and O'Grady (2012).

Phylogenetic species clustering

The geographic influence of the Hawaiian Islands on inter- and intra-island diversification in *Nesophrosyne* was examined by estimating the standardized mean phylogenetic distance (MPD) and the mean nearest taxon distance (MNTD), using PICANTE v1.3 in R (R Development Team, 2009; Kembel *et al.*, 2010). These statistics are equivalent to the net relatedness index (NRI) and nearest taxon index (NTI), respectively. The MPD measures the distance between taxon pairs through the root to the tips of a phylogenetic tree, generating an estimate of species clustering over the whole phylogeny. The MNTD measures clustering of terminal nodes. Negative values for both standardized values (MPD.z & MNTD.z) are evidence for species clustering, and a value ≥ 0 indicates phylogenetic evenness or overdispersion. The MPD and MNTD were estimated for the MCCT tree based on the 6-state biogeographic model described above. Outgroup and duplicate taxa were removed. Statistical significance was assessed by generating a null distribution of 1000 phylogenies with taxa randomly reshuffled at the tips.

Diversification rate analyses

Diversification dynamics in *Nesophrosyne* were inferred using R statistical packages APE and LASER (Paradis *et al.*, 2004; Rabosky, 2006; R Development Team, 2009). A lineage-through-time (LTT) plot was constructed from the MCCT with 95% confidence intervals derived from 1000 trees drawn from the Bayesian posterior distribution. Outgroup and duplicate taxa were removed from all trees. The constant rate (CR) test of Pybus and Harvey (2000) was used to investigate shifts in diversification rates. A γ-value less than -1.645 is significant to reject a constant rate hypothesis, indicating a rate slowdown (Pybus & Harvey, 2000). To deal with incomplete taxon sampling (*e.g.,* incomplete collections or extinction among lineages), Monte Carlo simulations of the CR test (MCCR) were conducted under step-wise increases of 5% missing taxa until statistical significance decayed (p-value > 0.01). Null distributions were simulated for 5000 phylogenies under an empirically determined rate-constant, pure-birth process for some number of species, with terminal nodes trimmed to the desired percent missing taxa. Phylogenetic uncertainty was assessed by the estimating the maximum γ-value for 1000 trees from the Bayesian posterior distribution.

Temporal shifts in diversification rates were estimated using birth-death likelihood (BDL) methods in LASER (Rabosky, 2006). BDL methods offer the opportunity to test *a priori* hypotheses of diversification rates shifts and provides superior power over the CR tests to detect shifts in diversification when background extinction is nonzero (Rabosky, 2006). The best fitting models were determined by estimating the likelihood of rate constant (RC) and rate variable (RV) models, and statistically evaluated by observing the difference in Akaike Information Criterion scores ($\Delta AIC_{RC} = AIC_{RC} - AIC_{RV}$). A positive ΔAIC_{RC} indicates support for the best-scoring rate variable model. Two rate constant models and four rate variable models were fit: i) pure birth (Yule), ii) constant-rate birth-death (B-D), iii) logistic diversity dependent (DDL), iv) exponential diversity dependent (DDX), v) a yule-2-rate model, and vi) a yule-3-rate model. Statistical significance was assessed by simulating a null distribution of 5000 trees under an empirically determined rate-constant pure-birth model for complete and incomplete sampling (50% and the 80% CR test breaking point). Phylogenetic uncertainty was addressed by calculating the ΔAIC_{RC} for 1000 trees drawn from the Bayesian posterior distribution. The null hypothesis of rate constancy was rejected if the observed ΔAIC_{RC} fell outside the 95% interval of the null distributions ($p < 0.01$).

RESULTS

Molecular dating

The Bayesian Maximum Clade Credibility Tree (MCCT) is presented in Figure 2, which has a similar global topology to that reported by Bennett and O'Grady (2012). Posterior support for relationships is moderate to high (PP = .75 – 1), with several mid-level nodes receiving low or no support (PP < 0.5; see Fig 2). Run-statistics for individual and combined MCMC runs, and the node ages for major clades are summarized in Supplementary Information (SI Tables 1 & 2). Inspection of the standard deviation of the uncorrelated log-normal relaxed molecular clock (std.UCLD) and covariance indicates rate heterogeneity among branches with no auto-correlation. The split between *Nesophrosyne* and *Orosius* is reconstructed as 4.5 Ma (95% HPD = 3.41 – 5.84 Ma), with the root divergence in *Nesophrosyne* at 3.2 Ma (95% HPD = 2.75 – 3.74

Ma). *Nesophrosyne* is split into two clades represented by nodes A and B (Fig. 2), with basal divergence within them at 2.93 Ma (95% HPD = 2.45 – 3.46 Ma) and 2.91 Ma (95% HPD = 2.46 – 3.35 Ma), respectively. The split dates of all other nodes are found in Fig. 2 and SI Table 2.

Ancestral range area and host-plant reconstructions

Performance of each biogeographic model varied by more than 2 log-likelihood units: UM-U = -358.8, UM-2 = -367.9, ST-U = -364.8, ST-2 = -375.3. The unconstrained UM-U and ST-U models yielded significantly lower likelihood scores, but gave high levels of uncertainty at basally diverging nodes that are geologically implausible (results not shown). Both models gave equally probable widespread ancestral ranges for the most basally diverging nodes (*e.g.,* the tree root and the root to Clade A, which diverged between 2.5 and 3.2 Ma) that include the youngest islands, Maui and Hawai`i, which did not emerge until 1.3 and 0.5 Ma, respectively. For this reason we reject the ST-U and UM-U. The UM-2 and ST-2 gave similar global results, with the UM-2 model giving a significantly better likelihood than the ST-2 (> 2 lnL units) and is shown in Fig. 3 (see SI Fig. 2 for ancestral ranges for all nodes). Both the UM-2 and ST-2 models gave a highly supported Kaua`i ancestor for the root node of *Nesophrosyne* (Prob = 0.77 – 0.81). The root node to Clade A was reconstructed as Kaua`i (Prob = 0.73 – 0.76); however, mid-level nodes gave high levels of uncertainty. The basally diverging nodes for Clade B gave a highly supported Kaua`i origin, with a mid-depth transition to O`ahu at highly supported internal nodes (Prob = 0.68 – 0.78, PP = 0.96 – 0.98). Ancestral range reconstructions for all other clades are found in Fig. 3 and SI Fig 1.

Ancestral host-plant reconstructions for all major clades are shown in Fig. 3. The ancestral state for the root node is equivocally split between Urticaceae (Prob = 0.40) and Rubiaceae (Prob = 0.38). The root node of Clade A is reconstructed as associated with Urticaceae (Prob = 0.98), with all subsequently diverging nodes receiving high support for Urticaceae association (Prob > 0.98). The ancestral host association for the root of Clade B is Rubiaceae, with subsequently diverging nodes receiving progressively higher support for a Rubiaceae association (Prob = 0.62 – 0.99). Within Clade A, host specific clades emerge from highly supported associations with Rubiaceae. See SI Fig. 2 for complete reconstructions.

Phylogenetic clustering

The MPD calculation indicated that only species on Kaua`i were significantly clustered (MPD.Z = -6.86, $p < 0.001$), while all other islands were slightly negative or positive and statistically insignificant (MPD.Z = -1.43 – 3.02, $p = 0.08 – .99$). MNTD demonstrated highly significant clustering on all islands (MNTD.Z = -4.83 – -6.67, $p = 0.001$) except Moloka`i and Lana`i (MNTD.Z = -1.05 – 0.34, $p = 0.1\ 4 – 0.63$). Full results for the phylogenetic clustering analyses are given in SI Table 3.

Diversification rate analyses

The lineage-through-time plot for the MCCT and the 95% confidence interval for 1000 post burn-in iterations is shown in Figure 2. Table 1 lists the results for diversification rate tests. The plotted distributions of observed and null distributions for the CR, MCCR, and ΔAIC_{RC} tests are shown in SI Fig. 3. The CR test γ-statistic is negative (CR = -8.48) and highly significant

when complete sampling is assumed ($p < 0.001$). This result is robust to MCCR simulations of incomplete sampling, with statistical significance beginning to decay at a threshold of 80% – 85% missing taxa (MCCR γ = -7.60 & -8.55, p = 0.002 & 0.06). Results are robust to phylogenetic uncertainty, with the max γ-value (-7.38) for 1000 posterior trees remaining statistically significant until a threshold of 75% missing taxa ($p > 0.01$).

The ΔAIC_{RC} test statistic for the MCCT significantly rejected constant rate diversification models ($p < 0.001$: Table 1), with the yule-3-rate model receiving the lowest AIC value over all multi-rate models. The yule-3-rate model approximated two transitions points occurring 0.63 Ma (st1) and 0.17 Ma (st2), with a step-wise speciation rate decline (r1 = 1.52, r2 = 0.62, & r3 = 0.03). Results are robust to phylogenetic uncertainty and incomplete sampling, with the ΔAIC_{RC} for the posterior distribution falling outside the simulated null distributions for complete and incomplete sampling (see SI Fig. 3).

DISCUSSION

Nesophrosyne*: An adaptive radiation model*

Examples of adaptive radiations in insects are few (Glor 2010) and no model exists for understanding how ecological and geographic forces drive diversification in sap-feeding insect groups. The *Nesophrosyne* system offers a geographically circumscribed radiation derived exclusively from *in situ* adaptation to the endemic Hawaiian flora, providing exceptional insight into the processes that drive adaptive diversification and community assembly (Glor, 2010). *Nesophrosyne* is the only leafhopper group to have diversified extensively on the archipelago, and has radiated widely to fill novel ecological opportunities in every climatic habitat type across the archipelago. This has led to high levels of local endemicity, cryptic diversity, host-plant specificity, and eco-morph evolution (Bennett & O'Grady, 2012).

Age and establishment of Nesophrosyne

Nesophrosyne presents a comparatively young group with a high species diversity, comprising over 190 species. *Nesophrosyne* split from *Orosius* 4.5 million years ago (3.41 – 5.84 Ma), with a subsequent basal divergence occurring 3.2 Ma (2.75 – 3.74 Ma). Ancestral range reconstructions support the initial colonization of Kaua`i, and a general progression-rule pattern of lineages geo-synchronously colonizing novel islands as they formed. This presents a scenario in which *Nesophrosyne* established on the Hawaiian Archipelago after the formation of Kaua`i, precluding the hypothesis of an older lineage descended from now subsided North West islands (Carson & Clague, 1995; Price & Clague, 2002), which has been found in few groups (Givnish *et al.*, 2009; Rubinoff & Schmitz, 2010). During this geologic period, Kaua`i was the highest and only ecologically mature sub-aerial landmass until the emergence of O`ahu (3.7 – 3.2 Ma), and was the most likely point of origin for colonizing lineages from the Western Pacific (Price & Clague, 2002).

Prior to Kaua`i, geologically driven extinction stripped the islands of most lineages and complex habitat structures (Whittaker *et al.*, 2008). This means that the current high elevation and wet forest biota either evolved *in situ* or re-colonized the islands after the formation of Kaua`i (Price & Clague, 2002). *Nesophrosyne* contains large clades of habitat specialists restricted to the endemic flora of high elevation wet forest ecosystems (*e.g.,* Clade A, Urticaceae

genera; and Clade M, Hydrangeaceae: *Broussaisia*), which did not exist early in the formation of the current high islands. Thus, the arrival times of endemic plant lineages and competing herbivores are expected to shape diversification and host specialization patterns. *Nesophrosyne* exhibits a general absence from the oldest and ecologically dominant plant lineages, including the silversword alliance (Asteraceae), lobelioids (Campanulaceae), and *Cyrtandra* (Gesneriaceae). The lobelioids and silverwords are estimated to have established prior to *Nesophrosyne* 13.6 Ma (10.49 – 16.71 Ma) and 5.2 Ma (4.4 – 6 Ma), respectively (Baldwin & Sanderson, 1998; Givnish *et al.*, 2009). Strikingly, these plant groups are dominant hosts for the endemic *Nesosydne* planthoppers (Delphacidae; Roderick & Percy, 2008), which is a large endemic herbivore group known to share the same basic niche as *Nesophrosyne* (*e.g.,* phloem feeding, oviposition in leaves, and nymphal development). This suggests the intriguing possibility that *Nesosydne* colonized Hawai`i prior to *Nesophrosyne* and preemptively excluded these niches.

Ecological and geographic isolation in Nesophrosyne *diversification*

Both ecological and geographic isolation are thought to drive the adaptive diversification of large species radiations (Gillespie, 2005; Losos, 2009; Goodman *et al.*, 2012). There is discussion of whether initial diversification is driven by ecological adaptation, or by early non-adaptive speciation with subsequent ecological specialization (Schluter, 2000; Rundell & Price, 2009). For Hawaiian phytophagous insects, high levels of host-plant specificity are common, but little is known about the phylogenetic patterns or mechanisms that drive these associations (reviewed in Roderick & Percy, 2008). The emergent diversification pattern in *Nesophrosyne* is due, at least in part, to novel ecological opportunity vis-à-vis endemic host-plant diversity and subsequent allopatric speciation between Hawai`i's volcanoes. Bennett and O'Grady (2012) reviewed patterns of host-plant use in the genus, and found that host associations are a major organizing force of extant species diversity, characterized by widespread, monophyletic species groups specialized to single host lineages (Bennett & O'Grady, 2011, 2012). Patterns of ancestral host-use detail a similar story. While the colonizing ancestor of *Nesophrosyne* is equivocally reconstructed as associated with the plant families Urticaceae and Rubiaceae, basal divergences in the genus are associated with *in-situ* ecological specialization to different plant families on Kaua`i. These host-associated divergences organized the genus into two parallel clades, A and B (see Fig. 2) defined by specialized associations: Clade A is strictly associated with genera in the family Urticaceae (except for *N. heopoko* on Hydrangeaceae), while Clade B forms a complex of host-specific clades in over twenty plant families derived from an ancestral association with Rubiaceae. Both plant families comprise multiple genera that are widely used hosts for *Nesophrosyne* species. Rubiaceae host genera include *Coprosma, Psychotria*, and *Hedyotus* (Clades C, F, & G); Urticaceae host genera include *Urera* and *Pipturus* (Clades A & P). Species in these genera represent diverse, ecologically dominant elements of the islands' mesic and wet-forest ecosystems (Wagner *et al.*, 1999), which are dominant habitats for *Nesophrosyne*.

The geologic structure of the Hawaiian Islands provides a scaffold for the diversification of *Nesophrosyne*. The genus comprises monophyletic groups with strict host associations that have species spread across each high island. General biogeographic patterns in these groups conform to a progression-rule pattern (Wagner & Funk, 1995); however, some more nested clades exhibit complex biogeography, resulting from highly supported back dispersals to older islands (*e.g.,* Clade E, Clade M, Clade P) and uncertainty in ancestral range inference (Clade A).

Phylogenetic clustering of species corroborates the highly dispersive nature of *Nesophrosyne*, with only Kaua`i showing significant clustering of species throughout the phylogeny. This may result from the origin and early restriction of *Nesophrosyne* to Kaua`i, or its geologically sustained relative isolation (128 km from the nearest large island versus a maximum of 48 km for other islands). Despite the lack of phylogenetic clustering on other islands, lineages show significant clustering at terminal nodes, indicating high levels intra-island diversification. This pattern is significant for all major high islands except Lana`i and Moloka`i, which have been historically connected to Maui (Price & Elliott-Fisk, 2004). This result reflects the fact that many host associated clades form complexes of allopatric cryptic sibling species ('allospecies': see Rundell & Price, 2009) with similar ecological associations distributed across each volcano and island (Bennett and O'Grady 2011, 2012). A similar pattern of ecologically decoupled allopatric divergence was found at the population level for nascent planthopper species (Delphacidae: *Nesosydne*) that specialize on *Dubautia* (Asteraceae; Goodman *et al.*, 2012). Taken together, these results indicate that the endemic flora permits colonization of other islands, while island geography imposes significant barriers to continued gene flow. These factors have led to the complex interplay between adaptive and non-adaptive processes in the diversification of endemic herbivorous insects groups, which is expected in species-rich radiations on complex geographic landscapes (Rundell & Price, 2009).

Host-plants clearly offer temporally dynamic ecological opportunity for phytophagous insect range expansion on Hawai`i. Several nested clades exhibit complicated biogeographic patterns associated with transitions to potentially recent establishments in the endemic Hawaiian flora: *Dodonaea* (Sapindaceae, Clade E), *Myoporum* (Scrophulariaceae, Clade D), and *Pipturus* (Urticaceae, Clade P). Arrival time has been estimated only for *D. viscosa*, which represents a young lineage that established on Hawai`i within the last 0.5 – 1.2 Ma (Harrington & Gadek, 2009). The emergence of *Nesophrosyne* species in Clade E is associated with specialization to *D. viscosa* and basal divergence on Maui approximately 1.2 Ma (0.99 – 1.50 Ma). The overlap in divergence dates for plant and insect suggests that transitions can happen rapidly. This relatively rapid transition is corroborated by comparisons between transition times in repeated, independent specialization of *Nesophrosyne* to other host-plant lineages: *Pipturus* (Urticaceae) on Kaua`i and O`ahu, 1.25 Ma (0.81 – 1.70 Ma: Clade A) and 1.10 Ma (0.88 – 1.34 Ma: Clade P), respectively; and *Broussaisia* (Hydrangeaceae) on Kaua`i and O`ahu, 1.70 Ma (1.22 – 2.22 Ma: *N. heopoko*, Clade A) and 1.67 Ma (1.40 – 1.95 Ma: Clade M), respectively.

Diversification dynamics of Nesophrosyne

Adaptive radiation theory predicts that novel ecological opportunities will lead to early bursts in speciation rates, but diversity-dependent slowdowns will follow as niches fill (Philmore & Price, 2008; Rabosky & Lovette, 2008a). Consistent with this hypothesis, *Nesophrosyne* exhibits an initially high species diversification rate that lasted from 3.2 – 0.67 Ma. The magnitude of the initial speciation rate (r1 = 1.51) is more than double the post-ceding slowdown (r2 = 0.62) and fifty times higher than the final rate (r3 = 0.03). The early rapid accumulation of lineages in *Nesophrosyne* is associated with host-plant transitions on Kaua`i, and coincides with geologic expansion of the Hawaiian Islands – the rapid growth of the multi-volcano islands O`ahu and Maui Nui (3.7 – 0.75 Ma; Carson & Clague, 1995). This finding suggests that *Nesophrosyne* established on Hawai`i during a period of open ecological opportunity that was

both expanding with the archipelago, and also maturing into complex geologically replicated habitat structures.

Nesophrosyne exhibits a strong signature of speciation rate decline, which is hypothesized as a diversity-dependent response to the exhaustion of ecological opportunity and geophysical area (Philmore & Price, 2008; Rabosky & Lovette, 2008a). The observed multi-rate diversification slowdown is tightly correlated with the formation of the youngest high islands, suggesting fundamental geologic controls on species diversity dynamics. The first rate decline occurred approximately 0.67 Ma and correlates to an island building pause after the formation of Maui Nui (0.8 Ma) and prior to the emergence of Hawai`i Island (0.5 Ma; Carson & Clague, 1995). The final speciation rate decline occurred 0.16 Ma, shortly after the formation of Mauna Loa (0.2 Ma), which marks the end point for the formation of current high islands (Carson & Clague, 1995). The geologically correlated, precipitous rate decline indicates that ecological opportunity, vis-à-vis host-plants, may have been exhausted by the time Maui fully formed. This further suggests that *Nesophrosyne* species may have reached equilibrium dynamics that are contemporarily controlled by island area (MaCArthur & Wilson, 1967). This hypothesis is supported by the fact that speciation in *Nesophrosyne* was initially rapid, and that the volcanoes of Maui and Hawai`i Islands comprise similar subsets of locally endemic, cryptic sibling species restricted to the same host lineages. Similar diversity-dependent declines and ecological similarity among young species have been found in other island groups (Gillespie & Baldwin, 2010; Rabosky & Glor, 2010).

Several important caveats apply to diversification rate analyses regarding potential sources of error, including low and taxonomically biased sampling, inability to quantify ephemeral species diversity, lineage specific rate heterogeneity, and unknown clade age and size (Philmore & Price, 2008; Cusimano & Renner, 2010; Rabosky, 2010; Rosenblum *et al.*, 2012). All of these factors remain difficult to assess for empirical datasets for novel groups of unknown size, especially large insect radiations. Our taxonomic coverage aimed to cover all major habitats across each volcano, targeted the distribution of native endemic plants, and included the known cryptic diversity in the genus. It is possible that our structured sampling violates the assumption of random sampling, leading to the overestimation of rate variability and decline in *Nesophrosyne* (Cusimano & Renner, 2010). However, our taxonomic sampling covers the known ecological and cryptic diversity in *Nesophrosyne*, which should buffer against overrepresentation of basally diverging lineages. This should also help to ameliorate some potential issues arising from typically undetected, young, or ephemeral species diversity (Rosenblum *et al.*, 2012), Moreover, the observed diversification slowdown in *Nesophrosyne* is robust to phylogenetic uncertainty and high levels of incomplete sampling based on our findings.

Extinction remains an important consideration when inferring diversification on islands, and is an explicit component of island biogeographic theory (MaCArthur & Wilson, 1967; Whittaker *et al.*, 2008; Ricklefs, 2010). Large extinction events are known to have occurred periodically on Hawai`i due to geologic processes and anthropogenic causes (Price & Clague, 2002; Gillespie *et al.*, 2008). Given the biased nature of anthropogenic extinction, effects may be lineage-specific, biasing interpretation of diversification results for endemic groups (Purvis, 2008; Rabosky, 2009). For *Nesophrosyne*, extensive human-mediated habitat degradation is likely to have affected extant species diversity through local extirpation and extinction of suitable hosts, including species in *Melicope* (Rutaceae), *Polyscias* (Araliaceae), and lobelioids (Campanulaceae; Wagner *et al.*, 1999). Despite this potential influence, our results indicate that extinction may not be a dominant force in the shape of *Nesophrosyne* diversity. It has been

suggested that historically high rates of extinction should erase evidence of early lineage accumulation (*e.g.* early adaptive or nonadpative radiations), leading to an abundance of young lineages and a corresponding increase in lineage diversification through time (Pybus & Harvey, 2000; Rabosky, 2006). This pattern is not observed in *Neosphrosyne*, where instead a strong signature of decline is detected. Nevertheless, diversification results for endemic Hawaiian radiations should be interpreted cautiously because extinction is unable to be accurately estimated both methodologically and empirically for endemic groups, and its overall effects will forever remain elusive (reviewed in Price, 2004; Rabosky, 2010).

Conclusion: A model for phytophagous insect diversification

Hawaii has long been a system of models for understanding adaptive radiation s and species diversification. Our study is the first to comprehensively assess how geology and ecology jointly shape the adaptive diversification dynamics in phytophagous insects not only on Hawai`i, but also for the suborder Auchenorrhyncha. These results construct a model for understanding the emergent macroevolutionary patterns in both groups. For large radiations that have diversified *in situ*, host availability, arrival times, and insect-insect competition shape overall diversification patterns. Once established, floristic diversity provides a temporally dynamic source of ecological opportunity, while the complex habitat structures promote allopatric divergence. Novel ecological opportunities lead to initially high speciation rates; however, rates will precipitously decline as ecological and geological niches are filled. Finally, our results provide a holistic approach that highlights the importance of ecological interactions, particularly in the diversification of native Hawaiian groups. In order to better understand the evolutionary patterns that govern community assembly on Hawai`i in particular, a general synthesis of the phylogenetic history, biogeography, and ecological diversity of the native biota would be invaluable.

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Tables

Table 1: Diversification rate test for the Constant-rate (CR) test and birth-death likelihood model-fitting test.

Pure Birth	Birth-death	DDL	DDX	Yule-2-rate	Yule-3-Rate	ΔAIC_{RC} *p*-value	γ-statistic MCCR Tests (*p*-value)
LH = - 609.09 ΔAIC = 80.82 r = 0.9365467	LH= - 609.09 ΔAIC = 82.82 r= .94 a= 0	LH = - 647.71 ΔAIC = 5.57 r1= 2.46 k = 210.13	LH = - 628.10 ΔAIC = 44.81 r1= 7.08 X = 0.45	LH= -640.21 ΔAIC = 22.58 r1= 1.51 r2= 0.44 st1= 0.63	LH = -653.50 ΔAIC = 0 r1 = 1.51 r2 = 0.62 r3 = 0.03 st1 = 0.63 st2 = 0.17	Yule-3-rate 80.82 Complete: $p < 0.001$ 50% missing taxa $p < 0.001$ 80% missing taxa $p = 0.0003$	*Nesohrosyne* MCCT -8.48 MCCR 80% missing taxa -7.61 ($p < 0.02$) MCCR 85% missing taxa -8.53 ($p = 0.06$)

Parameter abbreviations: r = speciation rate (corresponds to multiple speciation rates for multi-rate yule models, *e.g.,* r1); a = extinction rate; k = logistic ; x = exponential; st = transition for rates shifts in multi-rate yule models; ΔAIC = difference in Akaike Information Criterion score; MCCR = Monte Carlo Constant-Rate test (Critical γ-value).

Figures

Figure 1: The Hawaiian Archipelago. Island ages in millions of years (Ma) and taxon sampling are shown for each island. The inset graph shows the time-stratified island model implemented in LAGRANGE.

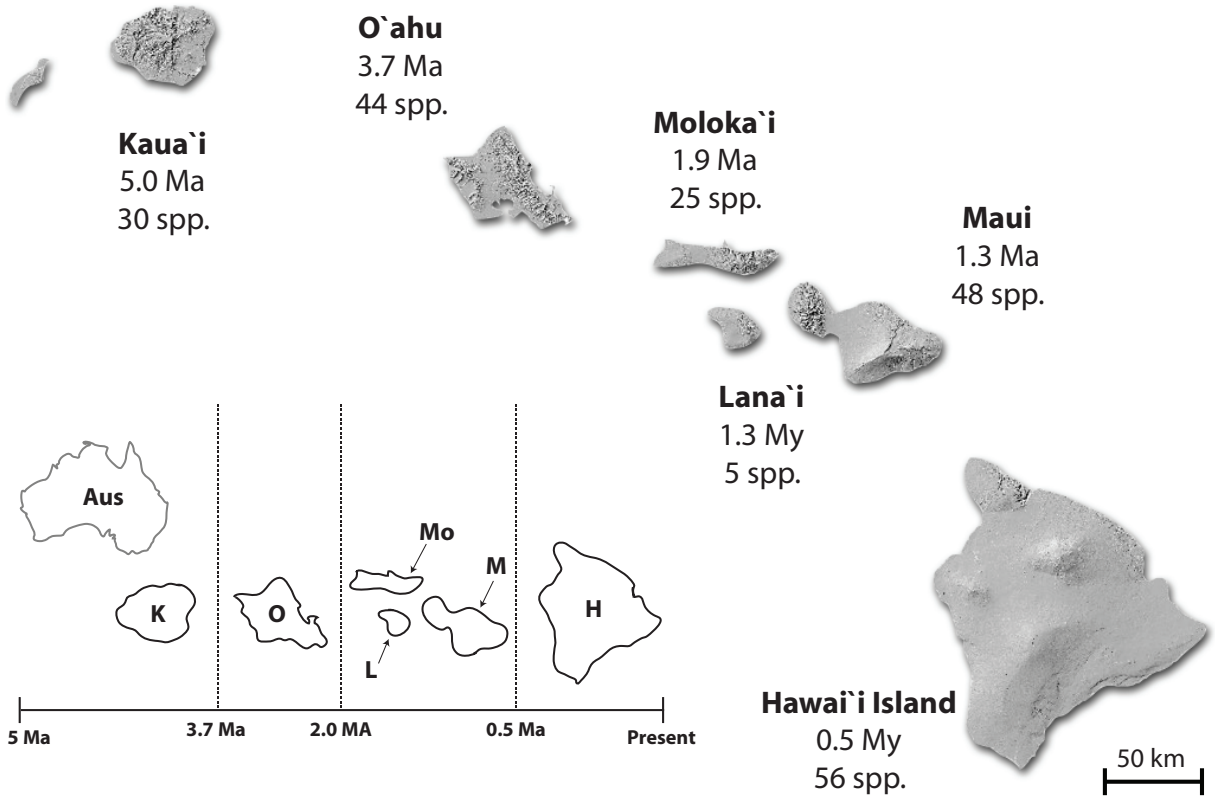


Figure 2: Time calibrated maximum clade credibility tree (MCCT) in millions of years for *Nesophrosyne* under a relaxed log-normal clock model (see SI Table 1 for summary statistics). Inset legend explains posterior node support, color-coded distributions for calibrated nodes, and species' island ranges. Blue bars show the 95% posterior density (HPD) for age estimates. Lettered boxes refer to clade names (see SI Table 2). Inset graph shows the log lineages-through-time plot for the MCCT. The red line represents the MCCT, with gray illustrating the 95% posterior distribution for 1000 Bayesian trees. Blue dashed lines illustrate the transition points for diversification rate declines for the yule-3-rate model (st1 and st2; See Table 1 for values).

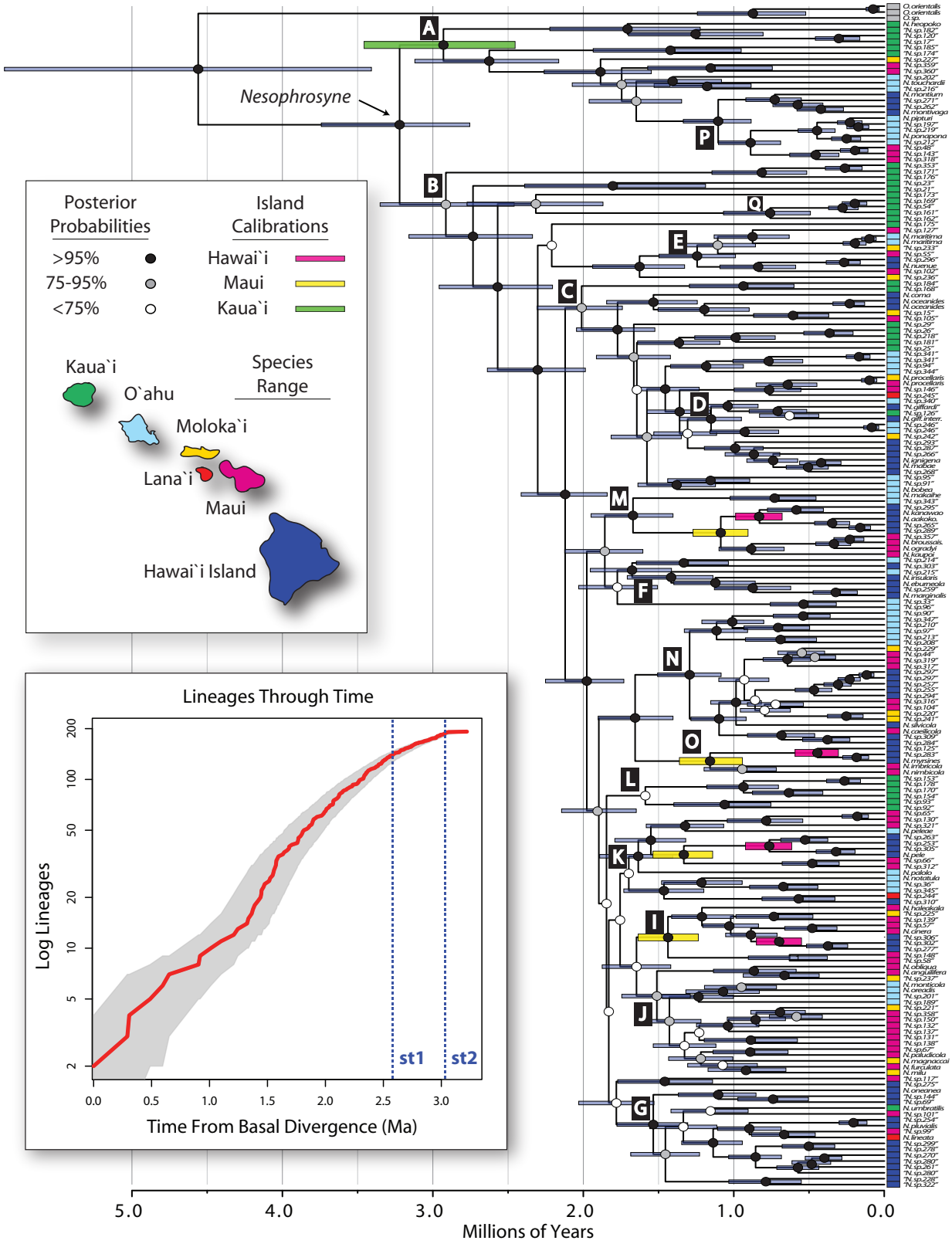
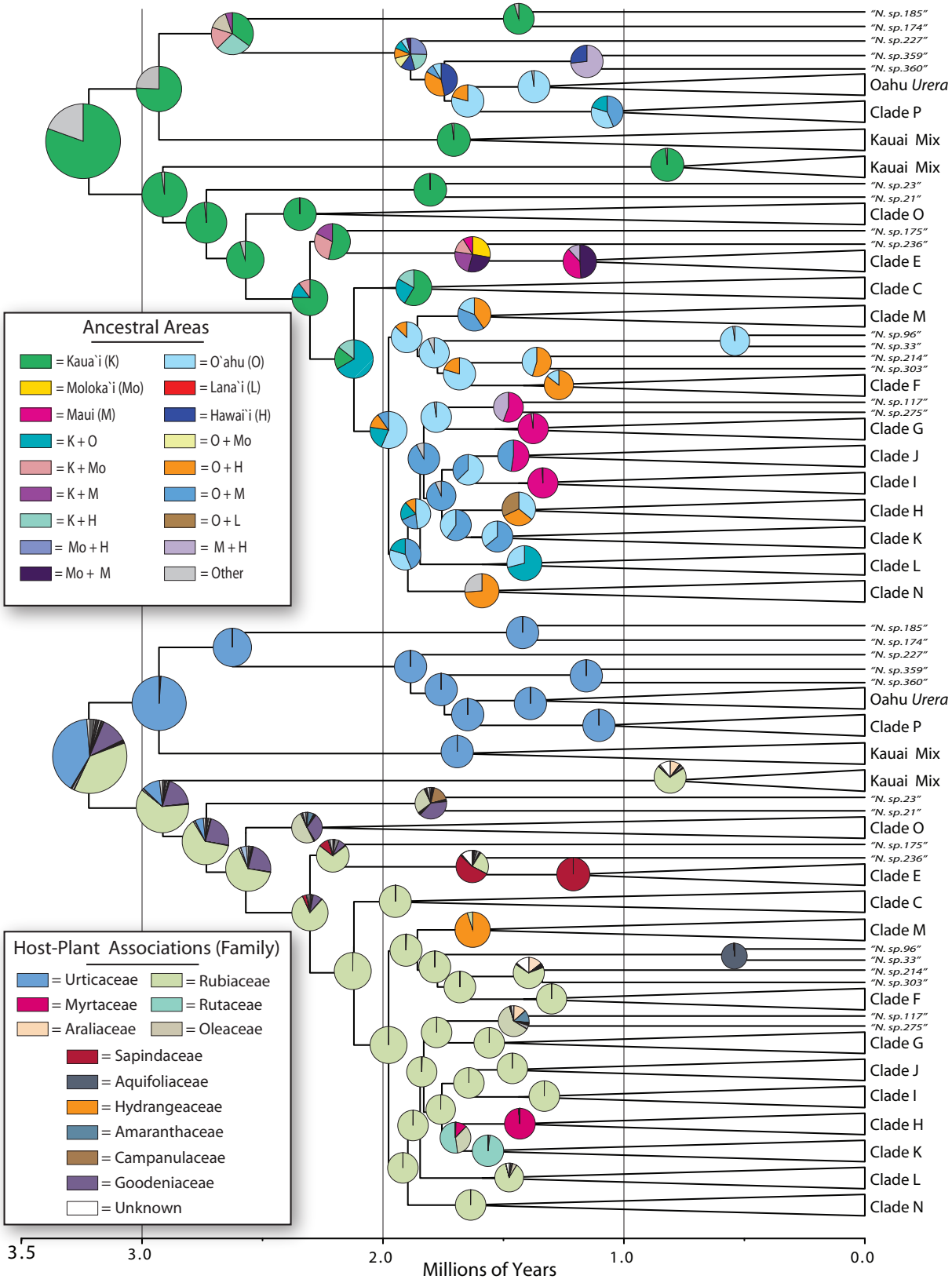
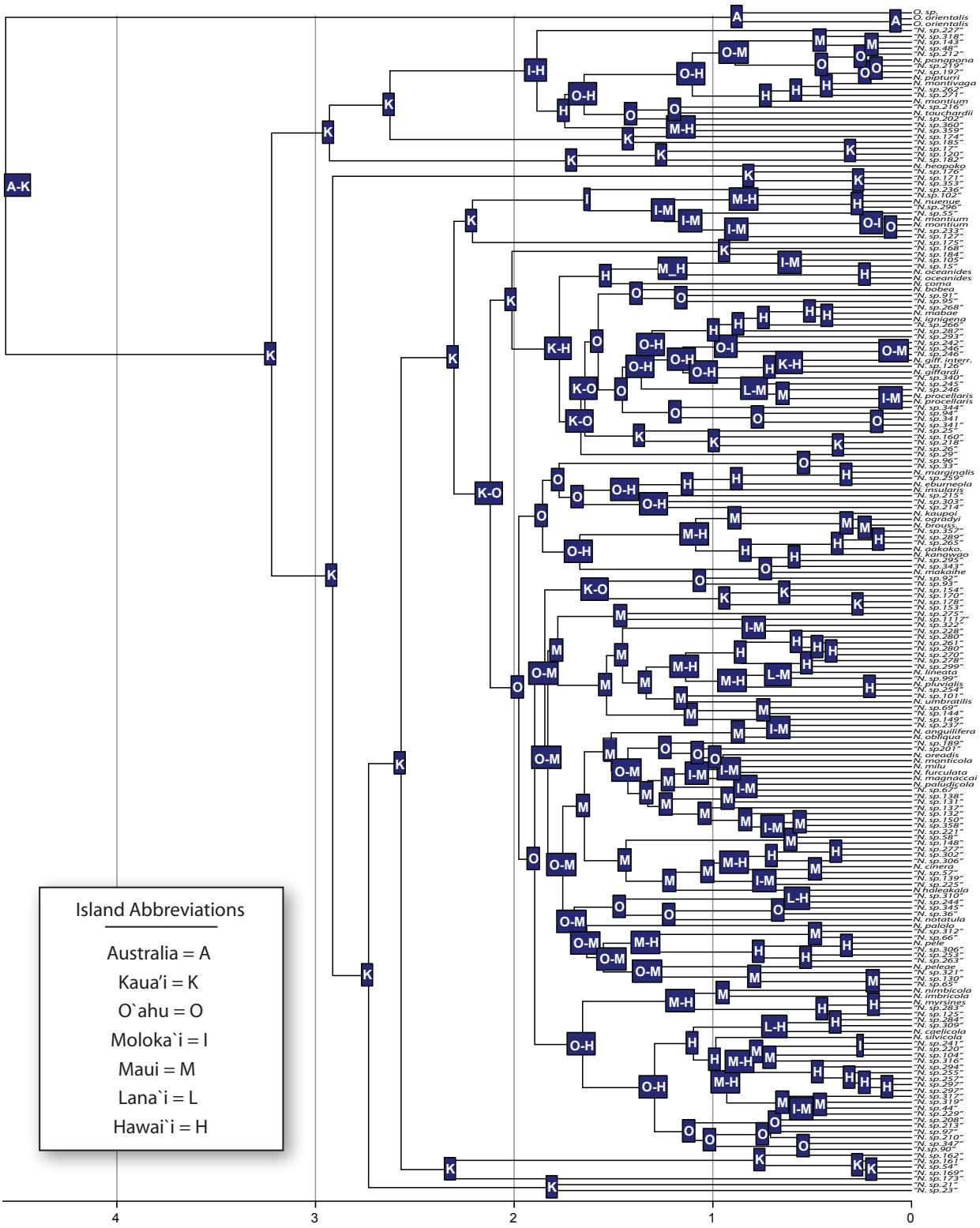

Nesophrosyne
A
B
C
D
E
F
G
I
J
K
L
M
N
O
P
Q
Posterior Probabilities
>95%
75-95%
<75%
Island Calibrations
Hawai`i
Maui
Kaua`i
Species Range
Kaua`i
O`ahu
Moloka`i
Lana`i
Maui
Hawai`i Island
Lineages Through Time
Log Lineages
2
5
10
20
50
100
200
st1
st2
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Time From Basal Divergence (Ma)
5.0
4.0
3.0
2.0
1.0
0.0
Millions of Years

Figure 3: Maximum likelihood ancestral state reconstructions. Outgroups have been trimmed. The top tree shows ancestral range reconstruction for the UM-2 model from LAGRANGE. The bottom tree shows host-plant ancestral state reconstructions. Pie charts illustrate relative probability for each state, and are color-coded according to state (see legend). Major clades have been collapsed to show overall patterns of ancestral states. Supplementary Figs. 1 & 2 show complete reconstructions for all nodes.

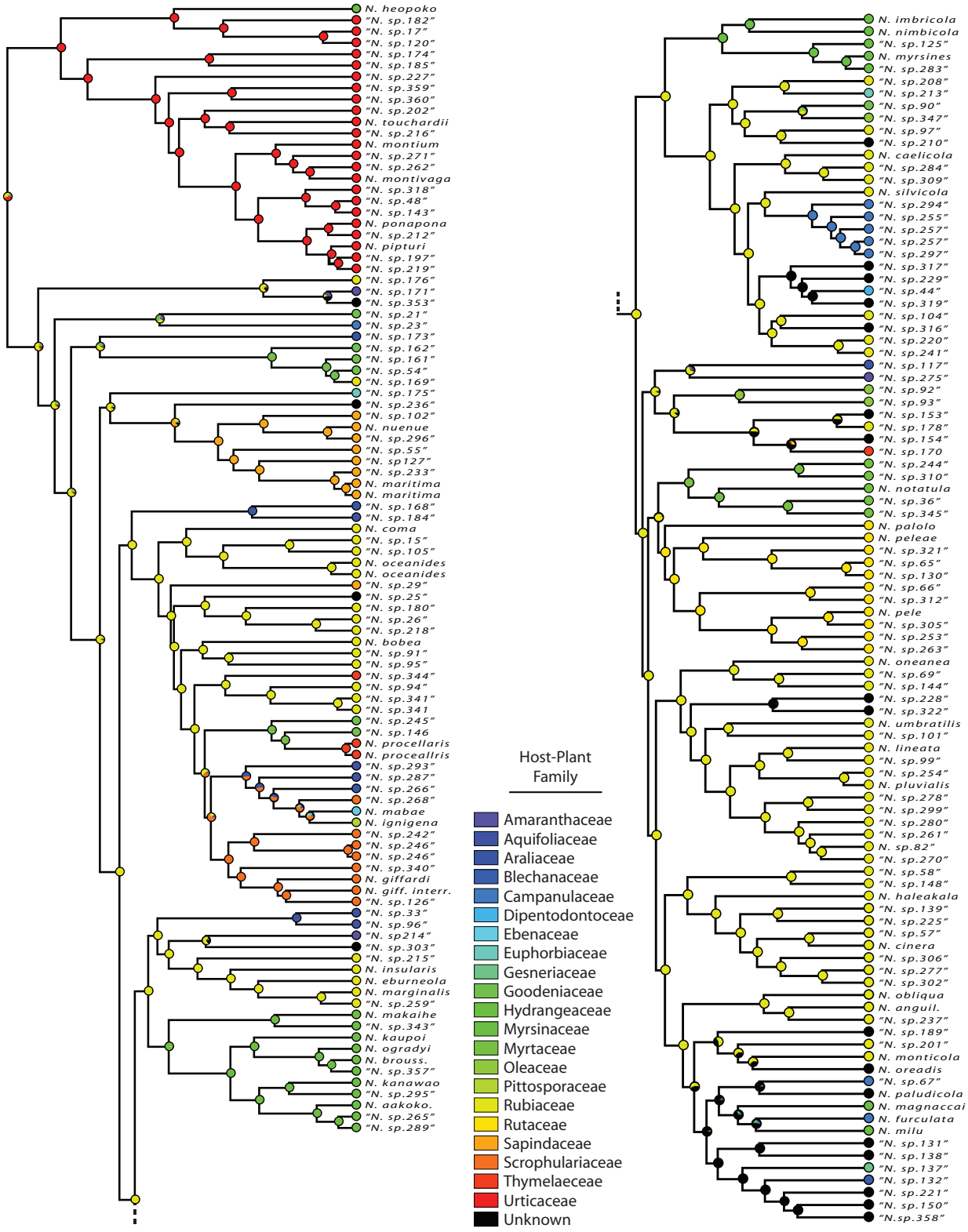

Ancestral Areas
= Kaua`i (K)
= O`ahu (O)
= Moloka`i (Mo)
= Lana`i (L)
= Maui (M)
= Hawai`i (H)
= K + O
= O + Mo
= K + Mo
= O + H
= K + M
= O + M
= K + H
= O + L
= Mo + H
= M + H
= Mo + M
= Other
Host-Plant Associations (Family)
= Urticaceae
= Rubiaceae
= Myrtaceae
= Rutaceae
= Araliaceae
= Oleaceae
= Sapindaceae
= Aquifoliaceae
= Hydrangeaceae
= Amaranthaceae
= Campanulaceae
= Goodeniaceae
= Unknown
"N. sp.185"
"N. sp.174"
"N. sp.227"
"N. sp.359"
"N. sp.360"
Oahu Urera
Clade P
Kauai Mix
Kauai Mix
"N. sp.23"
"N. sp.21"
Clade O
"N. sp.175"
"N. sp.236"
Clade E
Clade C
Clade M
"N. sp.96"
"N. sp.33"
"N. sp.214"
"N. sp.303"
Clade F
"N. sp.117"
"N. sp.275"
Clade G
Clade J
Clade I
Clade H
Clade K
Clade L
Clade N
3.5
3.0
2.0
1.0
0.0
Millions of Years

Supplementary Information Figures

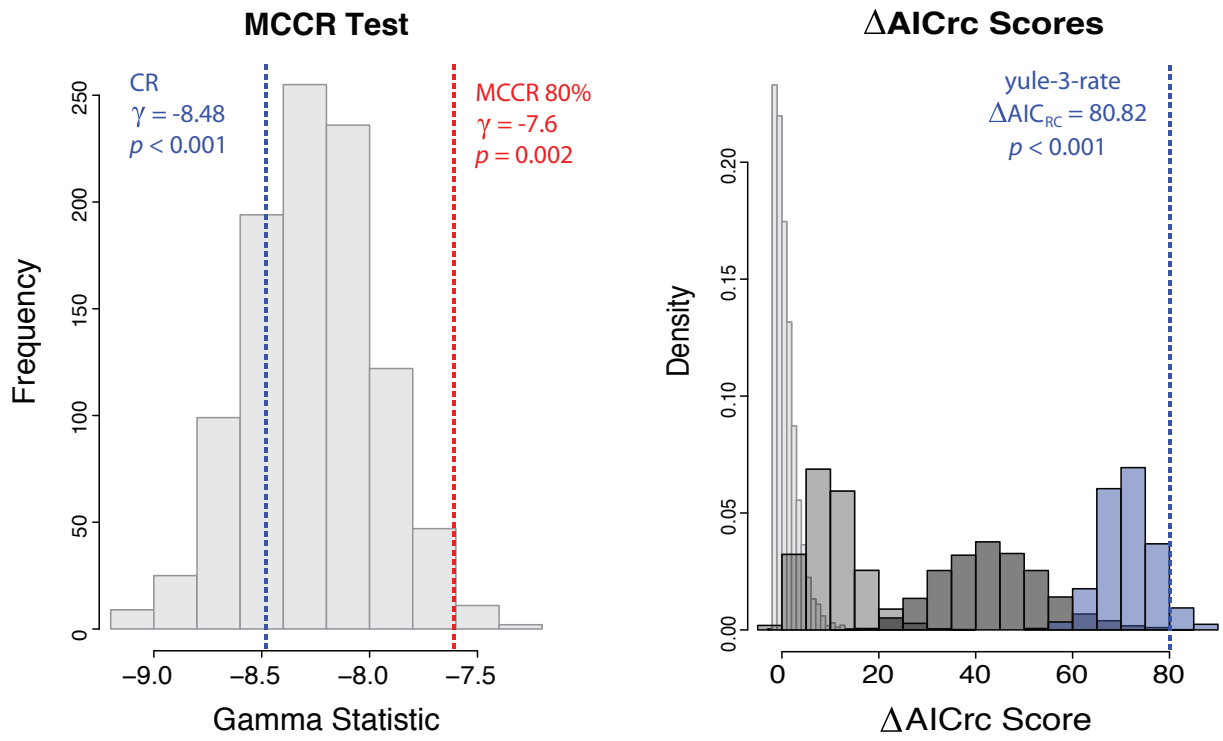
Supplementary Figure 1: Maximum likelihood ancestral range reconstructions for the un-collapsed MCCT from LAGRANGE. Node labels indicate historical island range, with abbreviations explained in the legend. Shown reconstructions represent the most probable ancestral ranges.


Island Abbreviations
Australia = A
Kaua'i = K
O`ahu = O
Moloka`i = I
Maui = M
Lana`i = L
Hawai`i = H
4
3
2
1
0

Supplementary Figure 2: Maximum likelihood ancestral host-plant reconstructions for the un-collapsed MCCT. Pie charts at nodes give relative probability of each state, corresponding to the color-coded legend.


N. heopoko
"N. sp.182"
"N. sp.17"
"N. sp.120"
"N. sp.174"
"N. sp.185"
"N. sp.227"
"N. sp.359"
"N. sp.360"
"N. sp.202"
N. touchardii
"N. sp.216"
N. montium
"N. sp.271"
"N. sp.262"
N. montivaga
"N. sp.318"
"N. sp.48"
"N. sp.143"
N. ponapona
"N. sp.212"
N. pipturi
"N. sp.197"
"N. sp.219"
"N. sp.176"
"N. sp.171"
"N. sp.353"
"N. sp.21"
"N. sp.23"
"N. sp.173"
"N. sp.162"
"N. sp.161"
"N. sp.54"
"N. sp.169"
"N. sp.175"
"N. sp.236"
"N. sp.102"
N. nuenue
"N. sp.296"
"N. sp.55"
"N. sp127"
"N. sp.233"
N. maritima
N. maritima
"N. sp.168"
"N. sp.184"
N. coma
"N. sp.15"
"N. sp.105"
N. oceanides
N. oceanides
"N. sp.29"
"N. sp.25"
"N. sp.180"
"N. sp.26"
"N. sp.218"
N. bobea
"N. sp.91"
"N. sp.95"
"N. sp.344"
"N. sp.94"
"N. sp.341"
"N. sp.341
"N. sp.245"
"N. sp.146
N. procellaris
N. proceallris
"N. sp.293"
"N. sp.287"
"N. sp.266"
"N. sp.268"
N. mabae
N. ignigena
"N. sp.242"
"N. sp.246"
"N. sp.246"
"N. sp.340"
N. giffardi
N. giff. interr.
"N. sp.126"
"N. sp.33"
"N. sp.96"
"N. sp214"
"N. sp.303"
"N. sp.215"
N. insularis
N. eburneola
N. marginalis
"N. sp.259"
N. makaihe
"N. sp.343"
N. kaupoi
N. ogradyi
N. brouss.
"N. sp.357"
N. kanawao
"N. sp.295"
N. aakoko.
"N. sp.265"
"N. sp.289"
Host-Plant Family
Amaranthaceae
Aquifoliaceae
Araliaceae
Blechanaceae
Campanulaceae
Dipentodontoceae
Ebenaceae
Euphorbiaceae
Gesneriaceae
Goodeniaceae
Hydrangeaceae
Myrsinaceae
Myrtaceae
Oleaceae
Pittosporaceae
Rubiaceae
Rutaceae
Sapindaceae
Scrophulariaceae
Thymelaeceae
Urticaceae
Unknown
N. imbricola
N. nimbicola
"N. sp.125"
N. myrsines
"N. sp.283"
"N. sp.208"
"N. sp.213"
"N. sp.90"
"N. sp.347"
"N. sp.97"
"N. sp.210"
N. caelicola
"N. sp.284"
"N. sp.309"
N. silvicola
"N. sp.294"
"N. sp.255"
"N. sp.257"
"N. sp.257"
"N. sp.297"
"N. sp.317"
"N. sp.229"
"N. sp.44"
"N. sp.319"
"N. sp.104"
"N. sp.316"
"N. sp.220"
"N. sp.241"
"N. sp.117"
"N. sp.275"
"N. sp.92"
"N. sp.93"
"N. sp.153"
"N. sp.178"
"N. sp.154"
"N. sp.170
"N. sp.244"
"N. sp.310"
N. notatula
"N. sp.36"
"N. sp.345"
N. palolo
N. peleae
"N. sp.321"
"N. sp.65"
"N. sp.130"
"N. sp.66"
"N. sp.312"
N. pele
"N. sp.305"
"N. sp.253"
"N. sp.263"
N. oneanea
"N. sp.69"
"N. sp.144"
"N. sp.228"
"N. sp.322"
N. umbratilis
"N. sp.101"
N. lineata
"N. sp.99"
"N. sp.254"
N. pluvialis
"N. sp.278"
"N. sp.299"
"N. sp.280"
"N. sp.261"
"N. sp.82"
"N. sp.270"
"N. sp.58"
"N. sp.148"
N. haleakala
"N. sp.139"
"N. sp.225"
"N. sp.57"
N. cinera
"N. sp.306"
"N. sp.277"
"N. sp.302"
N. obliqua
N. anguil.
"N. sp.237"
"N. sp.189"
"N. sp.201"
N. monticola
N. oreadis
"N. sp.67"
N. paludicola
N. magnaccai
N. furculata
N. milu
"N. sp.131"
"N. sp.138"
"N. sp.137"
"N. sp.132"
"N. sp.221"
"N. sp.150"
"N.sp.358"

Supplementary Figure 3: Histograms of the observed and null distributions for the diversity dynamics statistics. The histogram on the left (MCCR Test) shows the observed posterior distribution of γ -statistics for the 1000 post burn-in trees (gray). The blue line represents the γ-statistic for the MCCT, and the red line shows the critical γ-statistic for the MCCR test when 80% missing taxa is assumed. Statistical significance corresponds to the CR value of the MCCT. The histogram on the right (ΔAIC_{RC} scores) shows the distribution of ΔAIC_{RC} scores for the simulated null distributions assuming complete sampling (light gray), and 50% (medium gray) and 80% incomplete sampling (dark gray). The blue distribution represents 1000 Bayesian trees. The blue line shows the ΔAIC_{RC} score and statistical significance for the MCCT.



Supplementary Information Tables

Supplementary Table 1: Summary statistics for fully partitioned Bayesian molecular dating analyses. A 25% burn-in was applied individually to each run and for the combined runs.

MCMC Run	Mean -ln likelihood	ucld.stdev [95% HPD]‡	Covariance [95% HPD]	Mean Rate [95% HPD]	Yule Birth Rate [95% HPD]	Root Age § [95% HPD]	*Nesophrosyne* Age § [95% HPD]
1	-97837.6178	0.28 [0.24 – 0.32]	3.04E-2 [-6.63E-2 – 0.13]	2.08E-2 [1.81E-2 – 2.36E-2]	0.96 [0.79–1.15]	4.60 [3.37 – 5.79]	3.22 [2.74 – 3.73]
2	-97836.9186	0.28 [0.23 – 0.32]	3.09E-2 [-6.47E-2 – 0.13]	2.07E-2 [1.79E-2 – 2.34E-2]	0.96 [0.79–1.14]	4.56 [3.32 – 5.78]	3.23 [2.74 – 3.74]
3	-97836.9514	0.28 [0.24 – 0.32]	3.02E-2 [-6.65E-2 – 0.13]	2.08E-2 [1.80E-2 – 2.35E-2]	0.96 [0.79–1.14]	4.51 [3.36 – 5.75]	3.23 [2.75 – 3.73]
4	-97837.4962	0.28 [0.24 – 0.32]	2.97E-2 [-6.52E-2 – 0.13]	2.09E-2 [1.80E-2 – 2.36E-2]	0.96 [0.79–1.15]	4.58 [3.39 – 5.81]	3.22 [3.72 – 3.71]
Comb.	-97837.25	0.28 [0.24-0.32]	3.03 E-2 [-6.57E-2 – 0.13]	2.08E-2 [1.81E-2 – 2.36E-2]	0.96 [0.79–1.14]	4.56 [3.36 – 5.78]	3.22 [2.75 – 3.74]
ESS†	3389	2193	13982	5755	16957	2611	3974

§ Ages in millions of years

† Estimated Sampling Size

‡ 95% High Posterior Density

Supplementary Table 2. Node ages in millions of years (Ma) for combined Bayesian dating analysis done in BEAST.

Clade Name	Host-Plant Associations	Age Ma [95% HPD]
Orosius – Nesophrosyne Split		4.56
Nesophrosyne Root		3.22 [2.75 – 3.74]
A	Urticaceae	2.93 [2.45 – 3.46]
B	Other than Urticaceae	2.91 [2.46 – 3.35]
C	Mixed	2.01 [1.74 – 2.31]
D	Scrophulariaceae: *Myoporum*	1.51 [0.95 – 1.36]
E	Sapindaceae: *Dodonaea*	1.24 [0.99 – 1.50]
F	Rubiaceae: *Psychotria*	1.42 [1.13 – 1.71]
G	Rubiaceae	1.54 [1.31 – 1.78]
H	Myrtaceae: *Metrosideros*	1.46 [1.20 – 1.73]
I	Rubiaceae: *Coprosma*	1.44 [1.24 – 1.64]
J	Mixed	1.51 [1.29 – 2.74]
K	Rutaceae: *Melicope*	1.63 [1.41 – 1.90]
L	Kauai & Oahu Mixed	1.59 [NA]
M	Hydrangeaceae: *Broussaisia*	1.67 [1.40 – 1.95]
N	Mixed	1.29 [1.08 – 1.51]
O	Myrsinaceae: *Myrsine*	1.6 [0.94 – 1.36]
P	Urticaceae: *Pipturus*	1.10 [0.88 – 1.34]
Q	Goodeniaceae: *Sceavola*	2.32 [1.90 – 2.77]

Supplementary Table 3. Test statistics for phylogenetic and terminal level species clustering in PICANTE.

Mean Phylogenetic Distance (MPD)							
	ntaxa	mpd.obs	mpd.rand.mean	mpd.rand.sd	mpd.obs.rank	mpd.obs.z	mpd.obs.p
Hawaii	56	17.59	16.40	0.57	988.0	2.09	0.99
Kauai	30	11.33	16.43	0.79	1.0	-6.46	0.001
Lanai	5	15.40	16.49	2.45	333.0	-0.45	0.33
Maui	48	15.62	16.38	0.63	118.5	-1.21	0.12
Molokai	25	16.67	16.36	1.27	605.5	0.24	0.60
Oahu	44	16.32	16.38	0.66	466.0	-0.09	0.47
Mean Nearest Taxon Index (MNTI)							
	ntaxa	mntd.obs	mntd.rand.mean	mntd.rand.sd	mntd.obs.rank	mntd.obs.z	mntd.obs.p
Hawaii	56	2.75	4.41	0.25	1.0	-6.63	0.001
Kauai	30	3.03	5.79	0.46	1.0	-6.03	0.001
Lanai	5	12.20	11.40	2.36	637.5	0.34	0.64
Maui	48	3.23	4.75	0.30	1.0	-5.15	0.001
Molokai	25	6.47	7.65	0.95	104.5	-1.25	0.10
Oahu	44	2.50	4.91	0.32	1.0	-7.43	0.001

CHAPTER 4

Love, loss, and elevated evolutionary rates in the obligate co-primary β-proteobacterial endosymbionts of sap-feeding insects

Abstract

The evolutionary success of sap-feeding insects in the suborder Auchenorrhyncha is due to ancient associations with obligate bacterial endosymbionts, persisting for over 260 million years. Nearly all members of Auchenorrhyncha share the autapomorphic association with bacteriome restricted '*Candidatus* Sulcia muelleri', which has a transient association with evolutionarily diverse co-primary bacterial endosymbionts. This study examines the global relationships of the dual obligate, bacteriome restricted bacterial endosymbionts of the endemic Hawaiian leafhoppers (Cicadellidae: Deltocephalinae: *Nesophrosyne*), *Sulcia* and a novel β-proteobacterium in the '*Ca.* Nasuia' genus. A global phylogeny of 261 bacteria, including known endosymbionts associated with hosts from across the insect tree of life is reconstructed in order to understand the relationships of *Nasuia* in *Nesophrosyne* and related leafhoppers. The bacteriome association of *Nesophrosyne's* endosymbionts are investigated using Fluorescent *in situ* Hybridization techniques. Finally, absolute molecular rates are inferred for both endosymbionts to investigate rate changes in response to the adaptive radiation of their hosts. Results reveal a shared origin for the β-proteobacteria lineages throughout Deltocephalinae genera, and potentially throughout Auchenorrhyncha, suggesting an ancient history of acquisition and loss. Rates of evolution in both *Sulcia* and *Nasuia* in *Nesophrosyne* are highly elevated, and the fastest so far recorded. We propose a second species in the genus *Nasuia* to describe the novel β-proteobacterium in *Nesophrosyne*.

Key Words: Endosymbionts; β-proteobacteria; *Sulcia*; *Nasuia*; Deltocephalinae; *Nesophrosyne*

Introduction

Sap-feeding insects in the suborder Auchenorrhyncha (Hemiptera) are perhaps the most ecological dominant and terrestrially ubiquitous insect group on the planet (Schoonhoven *et al.*, 2005). Their evolutionary success is attributable, at least in part, to obligate interactions with bacterial endosymbionts (Moran, 2007; Baumann, 2005; Buchner, 1965). Extensive studies have revealed that these associations confer necessary nutritional biosynthetic pathways to supplement the nutritionally poor sap diet of their insect hosts (Baumann, 2005; Wu *et al.*, 2006; Moran *et al.*, 2009; McCutcheon *et al.*, 2009a; McCutcheon & Moran, 2010). The relationship between auchenorrhynchs and their endosymbionts are ancient, reaching back to their origin 260-280 million years ago (Moran *et al.*, 2005). Host-endosymbiont associations are considered to be a driving force in the adaptive diversification of the suborder, allowing lineages to dominant terrestrial plant life (Moran *et al.*, 2005).

Nearly all auchenorrhynchan families house the primary endosymbiont *Candidatus* Sulcia muelleri' except for Flatidae, Delphacidae, and Acanalonidae (Moran *et al.*, 2005; Note: we hereafter follow the single italicized naming convention for endosymbionts, *e.g., Sulcia*). Due to extreme genomic decay, *Sulcia* has lost some necessary biosynthetic functions, resulting in the acquisition of co-primary endosymbionts (CPE: Moran *et al.*, 2008; McCutcheon *et al.*, 2009a; McCutcheon & Moran, 2010). Some of the best-known CPE systems include the insect hosts cicadas ('*Candidatus* Hodgkinia cicadicola'; McCutcheon *et al.*, 2009a), Cicadellinae leafhoppers ('*Ca.* Baummania cicadellinicola'; Moran *et al.*, 2003), Cixiidae planthoppers ('*Ca.* Purcelliella pentastirinorum'; Bressan *et al.*, 2009), and Clastopteridae spittlebugs ('*Ca.* Zinderia

insecticola'; McCutcheon & Moran, 2010). The multitude of CPE associations suggests an evolving and dynamic relationship between hosts' nutritional needs, and obligate endosymbiont evolution. Genomic studies have revealed convergent biosynthetic function between these distantly related CPEs, complementary to pathways absent or lost in *Sulcia* (McCutcheon *et al.*, 2009a; McCutcheon & Moran, 2010). These studies have elucidated mechanisms of genome evolution, novel ecological interactions, and the potential evolutionary origins and success of insect herbivory (Moran, 2007; Moran *et al.*, 2005). The highly specialized lifestyles of CPEs have further led to extreme genomic decay (Wernegreen, 2002; Moran *et al.*, 2009; Nakabachi *et al.*, 2006) and elevated rates of molecular evolution (Bressan *et al.*, 2009; Moran *et al.*, 1995) in response to small population sizes, population bottlenecks, and bacterial mutational processes (Moran *et al.*, 2009; Nilsson *et al.*, 2005; Moran, 1996). Despite this deep knowledge, genomic and molecular studies have focused on particular taxonomic groups, leaving the broad-scale patterns of CPE associations across insect groups relatively unknown.

All members of the Cicadomorpha infraorder (cicadas, spittlebugs, planthoppers, treehoppers, and leafhoppers) are associated with *Sulcia* and a mosaic of CPEs localized within paired, bilateral organs called bacteriomes (Muller, 1962; Buchner, 1965). Bacteriomes comprise specialized, enlarged cells that house aggregates of endosymbiotic bacteria, which are segregated into different cell types (Muller, 1962; Buchner, 1965). The co-resident physiological association of *Sulcia* and CPEs may be linked to the mutualistic metabolic needs of both the host and endosymbionts, which have additionally lost some basic homeostatic biosynthetic functions (Wu *et al.*, 2006; McCutcheon & Moran, 2007, 2010), leading to a co-dependency between obligate endosymbionts (Bressan et al. 2009). Regardless, while CPEs are also relatively ancient and tailored to insect host and *Sulcia* needs (Takiya *et al.*, 2006; McCutcheon & Moran, 2010), their bacteriome localization acts as a revolving door with various bacterial lineages completely replacing others. The mechanisms behind this phenomenon are poorly understood, as is the overall diversity of CPE associations throughout Cicadomorpha.

Leafhoppers represent one of the largest cicadomorphan families (Zahniser & Dietrich, 2010). Leafhoppers rely on both phloem and xylem diets, and exhibit highly specialized associations with particular plant hosts (Zahniser & Dietrich, 2008). Like the rest of Cicadomorpha, leafhoppers have a conserved association with *Sulcia*, and varied associations with CPEs of different evolutionary origins (Noda *et al.*, 2012; Takiya *et al.*, 2006; Moran *et al.*, 2003). To date, only two leafhopper systems have been examined, including the genus *Nephotettix* (Deltocephalinae) associated with the β-proteobacterium *'Ca.* Nasuia deltocephalinicola', and sharpshooters (*e.g.,* Cicadellinae) associated with the γ-proteobacterium *Baumannia* (Noda *et al.*, 2012; Takiya *et al.*, 2006; Moran *et al.*, 2003; McCutcheon & Moran, 2010). The sharpshooter system has received extensive attention, elucidating its ancient nature, complementary metabolic role to *Sulcia*, and generally elevated but variable rates of molecular evolution (Wu *et al.*, 2006; McCutcheon & Moran, 2010; Takiya *et al.*, 2006; Moran *et al.*, 2005).The fact that both cicadellid subfamilies house co-primary endosymbionts of different origins indicates the dynamic evolutionary history between insect hosts and endosymbionts in leafhoppers. Despite the immense diversity of cicadellids, their endosymbiont associations remain almost entirely unknown, and no other groups have yet been examined.

This study focuses on the bacterial endosymbionts of the endemic Hawaiian leafhoppers (Cicadellidae: Deltocephalinae: *Nesophrosyne*) in order to expand our understanding of CPE associations throughout Auchenorrhyncha. *Nesophrosyne* is a relatively young, hyper-diverse genus of over 200 species endemic to Hawai`i that colonized the archipelago 3.2-4.5 million

years ago (Bennett & O'Grady, 2012a). Species in this genus have adaptively radiated to specialize on the endemic flora across the archipelago, exhibiting high levels of host-plant specificity (Bennett & O'Grady, 2012b, a). Bennett and O'Grady (2012a) identified the presence of a β-proteo CPE in native Hawaiian leafhoppers, found congruent cladogenetic relationships between endosymbiont and *Nesophrosyne*, and utilized the 16S locus for systematic analyses of host species-level relationships. Results from this study further demonstrated a close relationship between the β-proteo lineage in *Nesophrosyne* and in other members of the Deltocephalinae subfamily including the sister genus *Orosius* (both in the Opsiini tribe) and the distantly related *Deltocephalus* (Tribe: Deltocephalini), and hypothesized a relationship to the β-proteobacterium, *Nasuia*, found in *Nephotettix* (Tribe: Chiasmini; Noda *et al.*, 2012). Extensive screening of the Athsyanini tribe and related genera (*e.g., Scaphoideus*, *Osbornellus*, *Phlagotettix*, etc.) failed to recover the β-proteo lineage from other sections of the deltocephalinae subfamily (Bennett & O'Grady, 2012b). Thus, the origin and overall relationship between the Deltocephalinae subfamily and the β-proteobacteria remains uncertain.

The present study investigates the global taxonomic placement of the β-proteo CPE system in *Nesophrosyne*, and relationships between endosymbiont lineages in the Opsiini and Deltocephalini tribes to *Nasuia*. We use Fluorescent *in situ* Hybridization techniques to localize and confirm the bacteriome association of obligate endosymbionts in *Nesophrosyne*. Finally, the temporal evolutionary dynamics of the *Sulcia*–CPE system in *Nesophrosyne* are reconstructed in absolute time to understand the influence of adaptive radiation on the molecular evolution of associated endosymbionts. Results from this study broaden our understanding of CPE origins throughout Auchenorrhyncha, particularly within Cicadomorpha and the leafhoppers, all of which have intriguingly sustained interactions and loss of the β-proteobacterial CPE lineages.

Materials and Methods

Taxonomic and molecular sampling

Taxonomic sampling had a two-tiered focus aimed at i) inferring the global systematic relationships of the dual obligate endosymbionts in *Nesophrosyne*, and ii) estimating their molecular rates of evolution in absolute time. The data set for the global inference included a total of 261 taxa (see Supplementary Information [SI] Table 1 for complete taxonomic information of bacteria and hosts). All novel taxa included in this study are from the leafhopper subfamily Deltocephalinae, including 79 *Nesophrosyne*, 2 *Orosius*, and 1 *Deltocephalus* species. All other taxa included represent other endosymbionts and related environmental bacteria taken from Gruwell et al. (2010; and studies therein), and from GenBank Blast searches conducted with known *Nesophrosyne* endosymbiont sequences (See SI Table 1 for complete list of taxonomic sampling and associated citations). We included an additional 7 *Wolbachia* sequences from *Nesophrosyne* and related genera (Bennett *et al.*, 2012). *Sulcia* was sequenced for a subset of 28 species from across the phylogenetic and geographic distribution of *Nesophrosyne* for rate analyses (See SI Table 2). Fewer species were sequenced for *Sulcia* due to the relatively conserved nature of the 16S locus (average pairwise distance is approximately 99%). All specimens included in this study were field collected and preserved in either 95% ethanol for DNA sequencing, or acetone for Fluorescent *in situ* Hybridization experiments. All other genetic data were acquired from GenBank.

Sequencing and alignment

PCR primers and protocols were obtained from previous studies and optimized for *Nesophrosyne* (Table 1). The entire *Nesophrosyne* genus and outgroups from Bennett and O'Grady (2012a) were screened for the β-proteobacteria lineage; however, they used a shortened 1000 base pair section of the 16S locus for systematic analyses. For taxonomic identification, global phylogenetic inference, and molecular rate analyses of *Sulcia* and the β-proteobacterium in this study, we included only individuals sequenced for the entire 16S locus, achieved with the Eubacteria 10F + 1507R primers (β-proteo lineage: Moran *et al.*, 2003) and 10_CFB_FF + 1515R (*Sulcia* lineage: Moran *et al.*, 2005). PCR for each endosymbiont lineage was performed in a 25 µl reaction volume, containing 2.0 µl DNA template, 2.5 µl of 10 µM forward and reverse PCR primers, 0.5 µl of dNTP mix, 2.5 µl of 50 mM $MgCl_2$, 2.5 µl of 10× *i*Taq Buffer, 0.125 µl of *i*Taq (BIO RAD), and brought to volume with dH_20. PCR reaction conditions began with an initial 5 min denaturation step at 95°C; followed by a 35-cycle regime of 95°C for 30 sec, 56-60 °C for 45 sec (annealing optimized for each gene: Table 1), and 72°C for 1 min (extension phase); and, a final 72 °C extension for 5 min. Successful PCR amplifications were cleaned using the ExoSAP-IT protocol (USB). Both forward and reverse sequence directions were sequenced at the U.C. Berkeley, Barker Sequencing Center, using an ABI 3730 capillary sequencer. Sequences were submitted to GenBank for public archiving (SI Table 1).

Sequences of taxa were imported into GENEIOUS PRO v5.0.4 (Kearse *et al.*, 2012), which was used to build and edit contigs for forward and reverse sequence fragments. The identity of sequence data was checked using GenBank and the Ribosomal Database Project's (RDP) naive Bayesian taxonomic sequence classifier (Wang *et al.*, 2007). The global, *Sulcia*, and β-proteobacteria 16S datasets were aligned using the RDP 10, which informs alignment strategy with secondary ribosomal folding structure (Cole et al. 2008). Alignments were checked by eye for potential ambiguities. Global 16S alignments contained highly variable loop regions for which homologous statements among base pair (bp) positions were not trivial, and were removed from further analyses as follows: bp regions 96-127, 138-173, 847,893, and 1203-1337.

Phylogenetic and molecular rate estimation

Phylogenetic analyses were performed in a likelihood framework to i) examine the global phylogenetic placement of symbiont lineages in *Nesophrosyne* and related genera, and ii) infer the absolute rate of molecular evolution for *Sulcia* and the β-proteobacterium. A final molecular data matrix was assembled for 261 species and 1381 bp of the bacterial 16S locus. Likelihood models of base-pair evolution were determined for the global alignment and individual endosymbiont alignments for rate analyses, using the Bayesian Information Criterion in MODELTEST v3.7 (See Table 1: Posada & Crandall, 1998). A GTR+I+Γ model was selected for both the global and β-proteobacterium alignments, and a GTR+I was selected for the *Sulcia* alignment. Maximum likelihood criteria implemented in RAXML v7.2.7 on CIPRES (Stamatakis *et al.*, 2008; Miller *et al.*, 2009) were used to infer the global relationships of bacterial endosymbionts. RAXML is designed to handle large, complex datasets of the scope studied here (Stamatakis *et al.*, 2008). RAXML was run with the rapid bootstrapping algorithm under a GTRGAMMA model of nucleotide substitution for both 1000 bootstrap iterations and for reconstruction of the best-scoring maximum likelihood tree. The suggested default of 25 rate

categories was used. Resultant trees were exported into FIGTREE v1.3.1 for viewing and editing, and mid-point rooted.

The molecular rate of the 16S locus has been used in previous studies as a proxy for understanding the rate of evolution for bacterial endosymbionts (Moran *et al.*, 2009; Moran *et al.*, 1995; Bressan *et al.*, 2009). The molecular rate of evolution for *Sulcia* and the β-proteobacterium associated with *Nesophrosyne* were estimated in absolute time, using Bayesian phylogenetic methods in BEAST v1.7.2 on XSEDE in CIPRES (Drummond *et al.*, 2012; Miller *et al.*, 2009). BEAST implements a relaxed uncorrelated log-normal molecular clock model, which allows for the simultaneous estimation of molecular rates, rate heterogeneity (molecular clock), and correlation of rates among branches. Basal calibrations were applied with normally distributed probabilistic priors, with means centered on the inferred basal divergence for *Nesophrosyne* (3.223 Ma; Bennett & O'Grady, 2012a) and a confidence interval covering associated uncertainty (sd = 2.73-3.72 Ma). MCMC runs were conducted four independent times with a randomly generated starting tree under a Yule prior for 1×10^8 generations sampled every 4000th iteration. Convergence was assessed by plotting parameter estimates (*e.g.,* likelihood, mean rate, etc.) throughout the run and assessed with the Estimated Sampling Size (ESS > 200) in Tracer v1.5 (Rambaut & Drummond, 2007). The initial 25% of iterations for each run were discarded as burn-in, and all post-convergence runs were combined. Although the tree is not a parameter of interest, an MCCT was constructed for both runs from the combined last 5000 iterations of each run for quality assurance.

Whole specimen fluorescent in situ *hybridization*

In order to confirm the bacteriome association of the targeted bacterial lineages and their endosymbiont status, whole specimen Fluorescent *in situ* Hybridization visualization experiments were performed. The following fluorescing probes were designed for the aligned data matrices: Bet940 (5-TTAATCCACATCATCCACCG-3') for the β-proteobacterium labeled with 5' AlexaFlour-555 probe modification, and Sulc664R (5'-CCMCACATTCCAGMTACTCC-3') for *Sulcia* labeled with a 5'AlexaFlour-647 modification (Invitrogen).

FISH experiments were performed on 18 individuals for 5 species. Field collected specimens were stored in acetone, which was initially changed every few days. Specimens for FISH experiments were partially dissected in 80% EtOH by removing wings, legs, and perforating the dorsal side of the abdomen. Material was then fixed in Carnoy's solution (6 vol. 100% EtOH, 3 vo. Chloroform; 1 vol. Acetic Acid) and incubated at room temperature (RT) overnight. In order to reduce or eliminate autoflourescence of the exoskeleton and internal structures, specimens were rinsed (3× for 10 mins) and incubated in alcoholic hydrogen peroxide bleaching solution (6% H_20_2 - 80% EtOH) for 1-2 weeks until specimens appeared almost completely white (see Koga *et al.*, 2009). Material was then rinsed and stored in 100% EtOH at -20^o C until probe hybridization. Prior to FISH reactions, material was rehydrated in PBSTx (1 L PBS, 3 ml Triton X-100) by washing 3× for 10 min at RT, and rinsed with 500 ul hybridization buffer (2 ml of 1M Tris-HCl, 18 ml of 5M NaCl, 0.05 ml of 20% SDS, 30 ml Formamide, brought to 100ml with dH_20) for the same washing cycle. A hybridization-probe solution was mixed by combining 500 µl of the hybridization buffer, 5 µl of 10 µM concentrations of AL555-Bet940 and AL647-Sul664R probes, and 0.5 µl of 10 mg/ml DAPI counter-stain. Hybridization reactions were incubated overnight at RT with gentle agitation.

FISH imaging was done at the U.C. Berkeley Biological Imaging Facility, using a Zeiss LSM 710 laser scanning confocal microscope. Samples were prepared on depression slides in SlowFade AntiFade solution (Invitrogen). Initial images were done of the entire body to localize endosymbionts at 5× magnification. Further dissections were conducted as necessary to magnify the bacteriome at 20× magnification and bacteriocytes at 100× magnification.

Results

Phylogenetic relationships of bacterial endosymbionts

Phylogenetic and RDP classifier results confirm the β-proteobacteria classification of the CPE lineage in *Nesophrosyne* and bacteriodetes classification of *Sulcia*. The global phylogenetic relationships are presented in Fig. 1 (SI Fig.1 shows support values for all relationships). Phylogenetic placement of endosymbiont lineages from across the tree of life is congruent with previous studies (see Gruwell *et al.*, 2010). In general, global phylogenetic results reconstruct the major classes of bacteria and their corresponding endosymbionts with moderate to high support, including β-proteobacteria (*Nasuia*, *Vidania*, *Zinderia*, *Tremblaya*, and the CPE from *Nesophrosyne*: BS = 62), α-proteobacteria (*Wolbachia* (BS = 100), γ-proteobacteria (*Baumannia* from sharpshooters, *Carsonella*, etc.: BS = 99), and flavobacteria (*Sulcia*, *Uzinura*, *Brownia*, etc.: BS = 69).

Figure 1B shows the relationships between the co-primary β-proteo endosymbionts of Auchenorrhyncha (Cicadomorpha + Fulgomorpha). According to the sampling presented in this study, the β-proteobacteria endosymbionts from this group form a highly supported clade (BS = 94). The Auchenorrhyncha β-proteo clade is separated from other known endosymbiotic β-proteobacteria (*e.g., Tremblaya*) by basal divergences including an undetermined environmental bacterium (BS = 45), a *Diaphorina* (Psyllidae) associated bacteria (BS = 38), and environmental *Burkholderia* lineages (BS = 54-62). The basally diverging lineages in the Anchenorrhyncha clade include *Zinderia* (Host: Cicadoidea: *Clastoptera*; BS = 94) and then *Vidania* (Host: Fulgoroidea: *Hyalesthes*; BS = 100). The co-primary endosymbionts from the cicadellid Deltocephalinae subfamily form a highly supported clade (BS = 100). The results for cicadellid β-proteobacteria place those from *Nesophrosyne* and *Orosius* (both of the Opsiini tribe) sister with moderate support (BS = 53), while those of *Nesophrosyne* form a highly supported monophyletic clade (BS = 90). The β-proteobacteria of the genera *Deltocephalus*, *Matsumurattetix,* and *Nephotettix* are placed as a clade (BS = 95), with relationships between them poorly supported (BS = 35).

Molecular rate of 16S evolution

Molecular rate estimates from relaxed Bayesian dating methods are presented in Table 2. The resultant trees are available in supplementary information (SI Figs. 2 & 3). For further discussion, rates are converted to substitutions/site per 50 million years according to the convention of previous studies (see Moran et al. 1995; Bressan et al. 2005). Basal divergence times are consistent with those inferred for *Nesophrosyne* (see Table 2; Bennett & O'Grady, 2012a). Overall, the molecular rate for the β-proteo lineage is greatly elevated relative to *Sulcia*, and both are elevated compared to other studies (See Table 2 and Discussion). Examination of the standard deviation of the uncorrelated log-normal relaxed molecular clock (UCLD.stdev) and

Covariance reveal that the molecular rate of evolution in *Sulcia* is both clocklike (UCLD.stdev is abutting zero) and correlated among branches (covariance marginally negative). In contrast, the molecular rate of evolution in the β-proteo lineage is not clock-like (UCLD.stdev = 0.29), showing auto-correlated rate variation among branches.

Whole specimen fluorescent in situ *hybridization*

Whole specimen dissection and FISH images for female "*Nesophrosyne sp.23"* are presented in Figure 2. Investigation of all five species revealed similar morphology for bacteriome and bacteriocyte associated endosymbionts (data not shown). Dissected bacteriomes from freshly killed specimens are opaque, lacking any pigmentation (Fig. 2A). Both males and females contain elongate lobed, paired bilateral bacteriomes, extending through the second and third anterior abdominal segments. Individual bacteriomes comprise two structures of different cell types, each corresponding to a single endosymbiont (see Fig. 2A & B). Figure 2C illustrates the proximal, nested relationship between *Sulcia* (red) and the β-proteobacterium (green) in the bacteriome. Figure 2D and E show the two different bacteriocyte types and constituent bacteria. The bacteriocytes containing the β-proteo are a smaller (15-22 μm in diameter) single nucleated cell-type, and more uniform in shape than bacteriocytes housing *Sulcia*. The amorphous shape of the β-proteobacterium is observable within individual cells, which is congruent with previous studies (McCutcheon and Moran 2010; Noda et al. 2012). Bacteriocytes housing *Sulcia* are larger (45-56 μm in diameter), multinucleated, and with an irregular shape (Fig. 2E). The tubular, elongate shape of *Sulcia* is observable within the bacteriocyte (Fig. 2E). Finally, we were able to detect the β-proteobacterium within un-laid eggs dissected from the ovariole, providing evidence for the transovarial transmission of the β-proteobacterium (Fig. 3).

Discussion

Overview: Bacterial endosymbionts of Nesophrosyne *and Deltocephalinae leafhoppers*

The present study focuses on the broad-scale relationships of the endosymbionts found in the endemic Hawaiian leafhoppers and related genera in the cicadellid subfamily, Deltocephalinae. The dominant endosymbiont associations of *Nesophrosyne* include the hypothesized paired obligate bacteria, *Sulcia* and a co-primary β-proteobacterial endosymbiont. This bacterial complement is common among other sap-feeding insects (Moran *et al.*, 2008; Buchner, 1965; Baumann, 2005) and has been confirmed in the only other two leafhopper systems so far examined with molecular methods (Sharpshooters and the Green Rice Leafhopper; Moran *et al.*, 2003; Takiya *et al.*, 2006; Noda *et al.*, 2012; but see also, Wangkeeree *et al.*, 2011). The *Sulcia* lineage is thought to have been in association with sap-feeding insects for over 260-280 million years (Ma; Moran *et al.*, 2005), and has maintained a mosaic co-primary endosymbiont association for at least 200 Ma (McCutcheon & Moran, 2010). Our results further indicate a relatively conserved association with the β-proteobacterium throughout several genera in Deltocephalinae (Noda *et al.*, 2012). *Wolbachia* is also a common endosymbiont in *Nesophrosyne* species, and is likely to be found in most other deltocephaline leafhoppers (Bennett *et al.*, 2012). Transmission is thought to include extensive horizontal transfer between unrelated Hawaiian insects that share close ecological and host-plant

interactions. Unfortunately, the overall role and biological effects of *Wolbachia* on their Hawaiian hosts or other leafhoppers are currently unknown.

Deltocephalinae leafhoppers comprise the largest cicadellid subfamily, exhibiting a diversity of ecological and host-plant associations (Zahniser & Dietrich, 2008, 2010). Members are mono- and polyphagous phloem feeders, predominantly associated with grasses (Zahniser & Dietrich, 2008, 2010). As such, they represent the most economically important leafhopper group known, accounting for a disproportionate 77% (117 of 151) of all agriculturally important leafhopper species (see Zahniser & Dietrich, 2008). Both the evolution of this group and agricultural importance of these species are owed to their endosymbiont interactions. Their terrestrial and ecological ubiquity, and economic importance make deltocephalines a valuable model system for understanding endosymbiont-host evolution.

Relationships of β-proteobacteria within Deltocephalinae (Cicadellidae)

The β-proteobacterium found in *Nesophrosyne* is shared broadly across the Deltocephalinae subfamily, and has been named '*Candidatus* Nasuia deltocephalinicola' (Noda *et al.*, 2012). Phylogenetic results confirm the occurrence of the *Nasuia* lineage in the other leafhopper genera *Orosius*, *Matsumuratettix, Deltocephalus*, and *Nephotettix*. *Orosius* is the resolved sister genus to *Nesophrosyne* (both are placed within the tribe Opsiini; Bennett & O'Grady, 2012b), and the close relationships of their endosymbionts provides additional support for their common ancestry, and the origin of *Nesophrosyne* on the Hawaiian Archipelago. The other genera are from tribes distantly related to Opsiini, including Deltocephalini (*Deltocephalus* and *Matsumuratettix*) and Chiasmini (*Nephotettix;* reviewed in Zahniser & Dietrich, 2010). This widespread distribution of the *Nasuia* lineage throughout some Deltocephalinae tribes, as sampled here, provides strong evidence that *Nasuia* may be shared throughout the subfamily. However, Bennett and O'Grady (2012) were unable to recover the β-proteobacterium from ten genera in the Athsyanini tribe or that are taxonomically unplaced in other distantly related sections of the deltocephaline subfamily phylogeny (see Zahniser & Dietrich, 2010). While our results partially support the hypothesis of a conserved β-proteobacteria association throughout Deltocephalinae (Noda *et al.*, 2012), further sampling targeting the other known tribes and genera throughout the subfamily are required to confirm this.

The potentially shared origin and loss β-proteobacteria within Auchenorrhyncha

The *Nasuia* lineages are reconstructed as related to the co-primary β-proteobacteria "*Candidatus* Zinderia insecticola" associated with the spittlebug genus *Clastoptera* (Cercopoidea: Clastopteridae; McCutcheon & Moran, 2010), and '*Ca.* Vidania fulgoroideae' found in Fulgoroidea (Urban & Cryan, 2012). These relationships have been consistently recovered in other studies (Bressan *et al.*, 2009; Gruwell *et al.*, 2010; Noda *et al.*, 2012; Urban & Cryan, 2012). The Fulgomorpha and Cicadomorpha infraorders (Fulgoroidea + [Membracoidea + [Cicadoidea + Cercopoidea]]) comprise the monophyletic suborder Auchenorrhyncha (*sensu* Cryan & Urban, 2012). The phylogenetic placement of a shared β-proteobacterial lineage begs the question of whether or not the relationship is an autapomorphic association for the suborder that has been lost in some more derived groups (*e.g.,* cicadas). The split between Fulgoroidea and the lineage comprising the monophyletic Cicadomorpha (Cryan & Urban, 2012) occurred

between 200-260 Ma and appears to be dominated by β-proteo associations, spanning the root of Auchenorrhyncha and constituent superfamilies (McCutcheon & Moran, 2010; Scherbakov, 2002). Urban and Cryan (2012) estimated that the *Vidania* lineage may be as old as 200 million years, which is further consistent with the estimate by McCutcheon and Moran (2010) that *Sulcia* has had a co-primary endosymbiont (and possibly the β-proteobacterium, *Zinderia*) for the same length of time.

The shared origin hypothesis of the β-proteo-complex throughout Auchenorrhyncha has major implications, because it suggests that transitions to alternative bacterial classes have occurred at least and at different taxonomic levels within Cicadomorpha (*sensu* Cryan & Urban, 2012). First, Cicadoidea (the cicadas) are associated with the α-proteobacterium, '*Candidatus* Hodgkinia cicadicola', which shares the homologous, close bacteriome localization with *Sulcia* (McCutcheon *et al.*, 2009b). Second, the sharpshooter leafhoppers (*e.g.,* Cicadellidae: Cicadellinae) are associated with the γ-proteobacterium, *Baumannia* (Takiya *et al.*, 2006; Moran *et al.*, 2003). The evolutionary relationship between sharpshooters and *Baumannia* shows a long-term co-evolutionary relationship (Takiya et al. 2006), extending back 25-40 Ma based on appearance of the first fossils for the subfamily (Takiya *et al.*, 2006; Dietrich & Vega, 1995). Biological explanations for the potential transition away from β-proteobacteria may lie in the dietary habits of both cicadas and sharpshooters, which are xylem feeders (Novotny & Wilson, 1997; Brodbeck, 1993; White & Strehl, 1978). The ability to feed from xylem requires physical and metabolic compensations to overcome its negative tension (Zimmermann *et al.*, 1994) and increased nutrient deficiencies relative to phloem (Brodbeck, 1993; Novotny & Wilson, 1997). It has been demonstrated that compensatory adaptation to xylem has lead to morphological changes in both cicadas and sharpshooters (Novotny & Wilson, 1997). The acquisition of bacterial endosymbionts in alternative bacterial classes may offer the necessary mechanisms to overcome the nutritional deficiencies of a xylem diet (Takiya *et al.*, 2006). Novel associations with different proteobacteria may provide major adaptive evolutionary advantages for groups that have diversified extensively to feed from xylem. Despite this, spittlebugs are known xylem feeders that have maintained an association with β-proteobacteria lineage (Novotny & Wilson 1997). Thus, the overall role of diet in the maintenance of bacterial endosymbiont associations or transitions between them remains unclear.

While the phylogenetic evidence is intriguing for a conserved evolutionary history and loss of a β-proteobacterial lineage, the shared origin hypothesis for Auchenorrhyncha and Cicadomorpha should be interpreted cautiously. Current sampling is limited to representative lineages spanning large evolutionary time gaps and correspondingly long branch lengths, which are known to cause topological error in phylogenetic reconstructions (Felsenstein, 2004). Furthermore, convergent evolutionary genomic properties (*e.g.,* A-T bias, functional convergence, etc.) have been shown for several bacterial endosymbionts (McCutcheon *et al.*, 2009a; McCutcheon & Moran, 2010, 2007), including selective pressures that may bias phylogenetic reconstructions. The alternative explanation is multiple acquisition of β-proteobacteria in Auchenorrhyncha, suggesting a natural proclivity or genomic advantage conferred to sap-feeders through these convergent associations. Extensive broad-scale systematic and comparative genomic study is required to resolve this issue.

Molecular rates

The absolute rates of endosymbiont evolution for the 16S locus have been investigated in several other studies, including aphids, cixiid planthoppers, and cicadelline leafhoppers (Clark *et al.*, 2000; Takiya *et al.*, 2006; Bressan *et al.*, 2009). Direct estimates of rates in several of these groups were found to vary between 0.029 substitutions/site per 50 Ma in aphid-associated *Baumannia* (Clark *et al.*, 2000; Moran *et al.*, 1995), 0.045 substitutions/site per 50 Ma for *Purcelliella* in cixiid planthoppers, and 0.023 substitutions/site per 50 Ma for *Sulcia* in cixiids (Bressan *et al.*, 2009). Further evidence suggests a rapid rate of molecular evolution in the endosymbionts of the sharpshooter group (Takiya *et al.*, 2006). In *Nesophrosyne*, the inferred rates of molecular evolution for the obligate bacteriome-associated endosymbiont are greatly elevated. The absolute rate of evolution in *Sulcia* is 0.05 substitutions/site per 50 Ma, which is more than double the previous estimates. The *Nasuia* lineage exhibits a dramatically increased molecular rate of 0.95 substitutions/site per 50 Ma, making the *Nesophrosyne*-endosymbiont clade one of the fastest evolving obligate-endosymbiont systems so far recorded.

The dramatic rate increase of endosymbionts of *Nesophrosyne* is surprising, given the hypothesized long-term association of these endosymbionts, and their streamlined genomic functional and structural stability (McCutcheon & Moran, 2010; Moran *et al.*, 2005). Several biological explanations may account for the elevated rates. Bennett and O'Grady (2012b) demonstrated that *Nesophrosyne* is a relatively young adaptive radiation, exhibiting explosive early species diversification. Species in this genus have diversified extensively across a wide variety of endemic Hawaiian eudicot host-plants in over 20 families, which is likely to have changed the nutritional and metabolic requirements of both the host and endosymbionts. This history of host-plant shifts indicates that the adaptive radiation phenomenon, which has been invoked to explain rapid ecological diversification (Schluter, 2000), has consequences for both insect hosts and their endosymbiotic partners. While adaptive radiation appears to have elevated molecular evolutionary rates for both *Sulcia* and *Nasuia*, rates in *Sulcia* have been relatively constant, whereas the effect has been variable among *Nasuia* lineages in *Nesophrosyne* resulting in rate heterogeneity among lineages. This suggests an unequal effect on the endosymbionts during the adaptive diversification of *Nesophrosyne*. This difference may be a consequence of the nutritional and metabolic role *Nasuia* plays relative to *Sulcia.* Unfortunately, limited taxonomic sampling in other cicadellid groups currently prohibits comparative rate analyses to test this hypothesis more broadly.

Conclusion: Taxonomy and biology of the novel β-proteo in Nesophrosyne

FISH microscopy confirms the localized restriction of *Sulcia* and the co-primary β-proteobacterium to the bacteriome and bacteriocytes homologous across Cicadomorpha (Muller, 1962; Buchner, 1965; McCutcheon & Moran, 2010; Moran *et al.*, 2005). Noda et al. (2012) recently described the β-proteobacterium as "*Candidatus* Nasuia" from *Nephotettix cinticeps* (the green rice hopper) and *Matsumuratettix hiroglyphicus*, and suggested that the lineage may be found throughout Deltocephalinae. The evolutionary relationships and restriction of the *Nasuia* lineage to bacteriocytes in *Nesophrosyne* is taken as further evidence for its un-culturable nature consistent with the *Candidatus* naming convention used previously, which we retain (Murray & Schleifer, 1994). Given the highly supported shared origin for the Deltocephalinae β-proteobacterium, molecular divergence between the *Nasuia* lineage in *Nesophrosyne* and

Nephotettix (average pairwise distance = 88.6%), lack of pigmentation, rapid evolutionary rates, and insular nature of the host genus *Nesophrosyne*, we propose a second species to the '*Ca.* Nasuia' genus: '*Candidatus* Nasuia zimmermania', named after the esteemed Hawaiian entomologist Elwood C. Zimmerman, whose visionary work on the Hawaiian entomofauna and *Insects of Hawaii* volumes (Univ. Haw. Press) has been an inspiration. The proposed name corresponds to the β-proteobacterium in the leafhopper genus *Nesophrosyne*, with the reference strain from *N. aakokohaikea* Bennett, specializing on native Hawaiian Hydrangeaceae (*Broussaisia arguta*) and endemic to Hawai`i Island. Unique 16S sequence for *Nasuia zimmermania* are 5'-CACAAGCGGTGGATGATGTGGATTAATTCGATG-3' (position 904-936 *Escherichia coli*), and 5'-AGCTCGTGTCGTGAGATGTTGGGTTAAGT-3' (*E. coli* position 1039-1189).

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Tables

Table 1: Polymerase Chain Reaction and Fluorescent *in situ* Hybridization oligonucloetides for *Sulcia* and the β-proteobacterium, *'Ca.* Nasuia deltocephalinicola'.

Gene	Primer Name (Annealing Temp.)	Primer Sequence 5'-3'
Sulcia 16S	10F[#] (56°)	CCGGTYTGAACTCARATCA
	1507R[#]	CRMCTGTTAWCAAAAACAT
	16S FISH Probe: Sulc664R[*]	CCMCACATTCCAGMTACTCC
Nasuia β-proteobacteria 16S	10F[¢] (58°)	AGTTTGATCATGGCTCAGATTG
	1507R[¢]	TACCTTGTTACGACTTCACCCCAG
	Beta_300[*] (60°)	GCGGATGTATAGCCACACTGGGA
	Beta_1352[*]	ATAAAACTTACTCCCATGGTG
	16S FISH Probe: Bet940[*]	TTAATCCACATCATCCACCG

[¢] Moran *et al.* 2003, 2005
[*] Primers designed in this study

Table 2: Summary statistics for Bayesian molecular rate of evolution inferred with BEAST. A 25% burn-in was applied individually to each run (1-4) and then combined (comb.).

MCMC Run	Mean -ln likelihood	ucld.stdev [95% HPD]‡	Covariance [95% HPD]	Mean Rate [95% HPD]	Root Age [95% HPD]
			***Nasuia* β-proteobacterium**		
1	-8440.10	0.29	-1.0E-2	1.90E-2	3.13
		[0.16 – 0.42]	[-0.16 – 0.16]	[1.36E-2 – 2.48E-2]	[2.54 – 3.73]
2	-8439.65	0.29	6.84E-4	1.90E-2	3.14
		[0.17 – 0.42]	[-0.16 – 0.16]	[1.37E-2 – 2.48E-2]	[2.55– 3.74]
3	-8440.44	0.29	9.36E-4	1.90E-2	3.14
		[0.27 – 0.42]	[-60.15 – 0.17]	[1.35E-2 – 2.46E-2]	[2.53 – 3.72]
4	-8440.03	0.29	-0.03E-2	1.90E-2	3.14
		[0.16 – 0.41]	[-0.16 – 0.16]	[1.38E-2 – 2.50E-2]	[2.53 – 3.72]
Comb.	-8440.05	0.29	5.89E-5	1.90E-2	3.14
		[0.16 – 0.41]	[-0.16 – 0.16]	[1.36E-2 – 2.48-2]	[2.55– 3.73]
ESS	8792.96	8830.10	70543.32	29360.41	74537.37
			Sulcia		
1	-2149.97	0.38	-1.42E-2	9.67E-4	3.14
		[7.80E-6 – 1.03]	[-0.28 – 0.26]	[4.27E-4 – 1.59E-3]	[2.55 – 3.74]
2	-2151.08	0.39	-1.51E-2	9.80E-4	3.14
		[3.52E-5 – 1.05]	[-0.28 – 0.26]	[4.32E-4 – 1.62E-3]	[2.53 – 3.73]
3	-2149.94	0.38	-1.33E-2	9.74E-4	3.13
		[4.60E-5 – 1.04]	[-0.27 – 0.26]	[4.41E-2 – 1.62E-3]	[2.54 – 3.73]
4	-2149.91	0.37	-1.18E-2	9.71E-4	3.14
		[2.71E-6 – 1.00]	[-0.28 – 0.26]	[4.48E-4 – 1.67E-3]	[2,52 – 3.71]
Comb.	-2150.26	0.38	-1.34E-2	9.78E-4	3.14
		[2.71E-6 – 1.03]	[-0.28 – 0.26]	[4.37E-4 – 1.62E-3]	[2.54 – 3.73]
ESS†	593.81	47790.41	73950.3	11458.81	75004

† Estimated Sampling Size
‡ 95% High Posterior Density

Figures

Figure 1: Maximum likelihood phylogeny of bacterial endosymbiont lineages inferred from the 16S locus, showing the placement of the β-proteobacterium associated with Deltocephalinae leafhoppers included in this study. A. Global placement of the endosymbiont lineages among bacterial classes and relationships to other known endosymbionts. Bacteria classes are colored and labeled. Blue shows the placement of the Deltocephalinae β-proteobacterial endosymbionts, *Zinderia*, *Vidania*, and *Nasuia*. B. Relationships for Deltocephaline leafhopper genera and other β-proteobacteria associated with Auchenorryhncha (Cicadomorpha + Fulgomorpha; *sensu* Cryan and Urban 2012), which form a highly supported clade. Numbers above branches correspond to bootstrap support values.

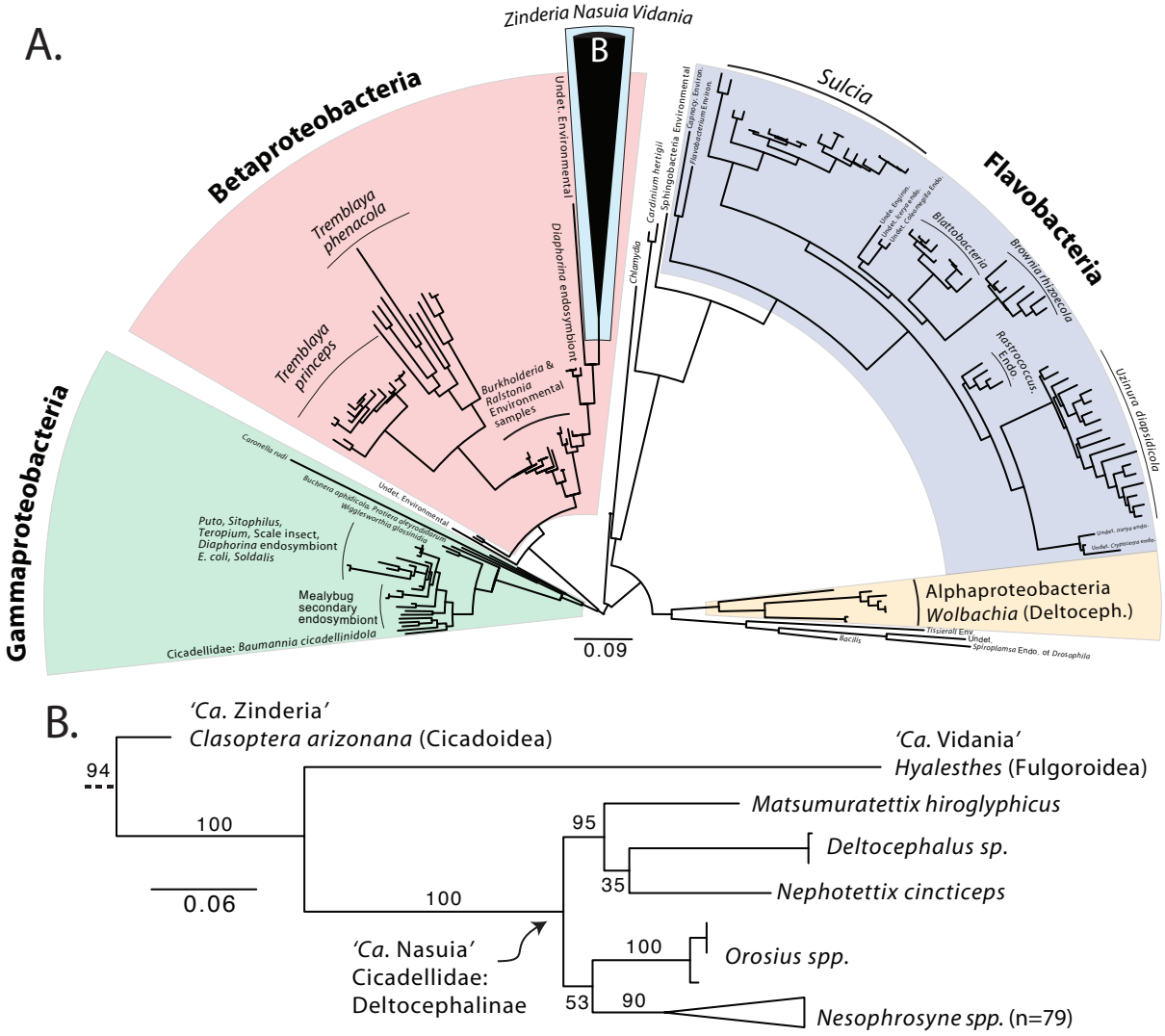

A.
Zinderia Nasuia Vidania
B
Betaproteobacteria
Undet. Environmental
Diaphorina endosymbiont
Tremblaya phenacola
Tremblaya princeps
Burkholderia & Rolstonia Environmental samples
Sulcia
Flavobacteria
Cardinium hertigii
Sphingobacteria Environmental
Blattabacteria
Brownia rhizoecola
Uzinura diaspidicola
Gammaproteobacteria
Carsonella ruddi
Buchnera aphidicola, Protease oligopeptidase, Wigglesworthia glossinidia
Undet. Environmental
Puto, Sitophilus, Teropium, Scale insect, Diaphorina endosymbiont E. coli, Soldalis
Mealybug secondary endosymbiont
Cicadellidae: Baumannia cicadellinidola
Alphaproteobacteria
Wolbachia (Deltoceph.)
Bacilli
Undet. Spiroplasma Endo. of Drosophila
0.09
B.
'Ca. Zinderia'
Clasoptera arizonana (Cicadoidea)
'Ca. Vidania'
Hyalesthes (Fulgoroidea)
Matsumuratettix hiroglyphicus
Deltocephalus sp.
Nephotettix cincticeps
Orosius spp.
Nesophrosyne spp. (n=79)
'Ca. Nasuia'
Cicadellidae:
Deltocephalinae
94
100
95
35
100
100
53
90
0.06

Figure 2: Microscopy and whole specimen Fluorescent *in situ* Hybridization (FISH) imaging of the obligate bacteriome-associated bacterial endosymbionts of "*Nesophrosyne sp.23"*. All images are of the venter. FISH imaging shows '*Candidatus* Sulcia muelleri' (Red), the β-proteobacterium *'Ca.* Nasuia deltocephalinicola' (Green), and host insect tissues counterstained with blue. A & B. The ventral habitus with legs and abdominal plates dissected to reveal the opaque bacteriomes (inset image is an enlarged bacteriome), and FISH results demonstrating the paired bacteriomes and associated bacteria, respectively. C. The left lateral bacteriome at 20× magnification at the anterior end of the abdomen, highlighting the segregated, nested association of each bacteria in different bacteriocytes. D & E. The individual bacteriocyte types, housing both *Nasuia* (D) and *Sulcia* (E) at 100× magnification. Bars associated with each figure correspond to absolute size.

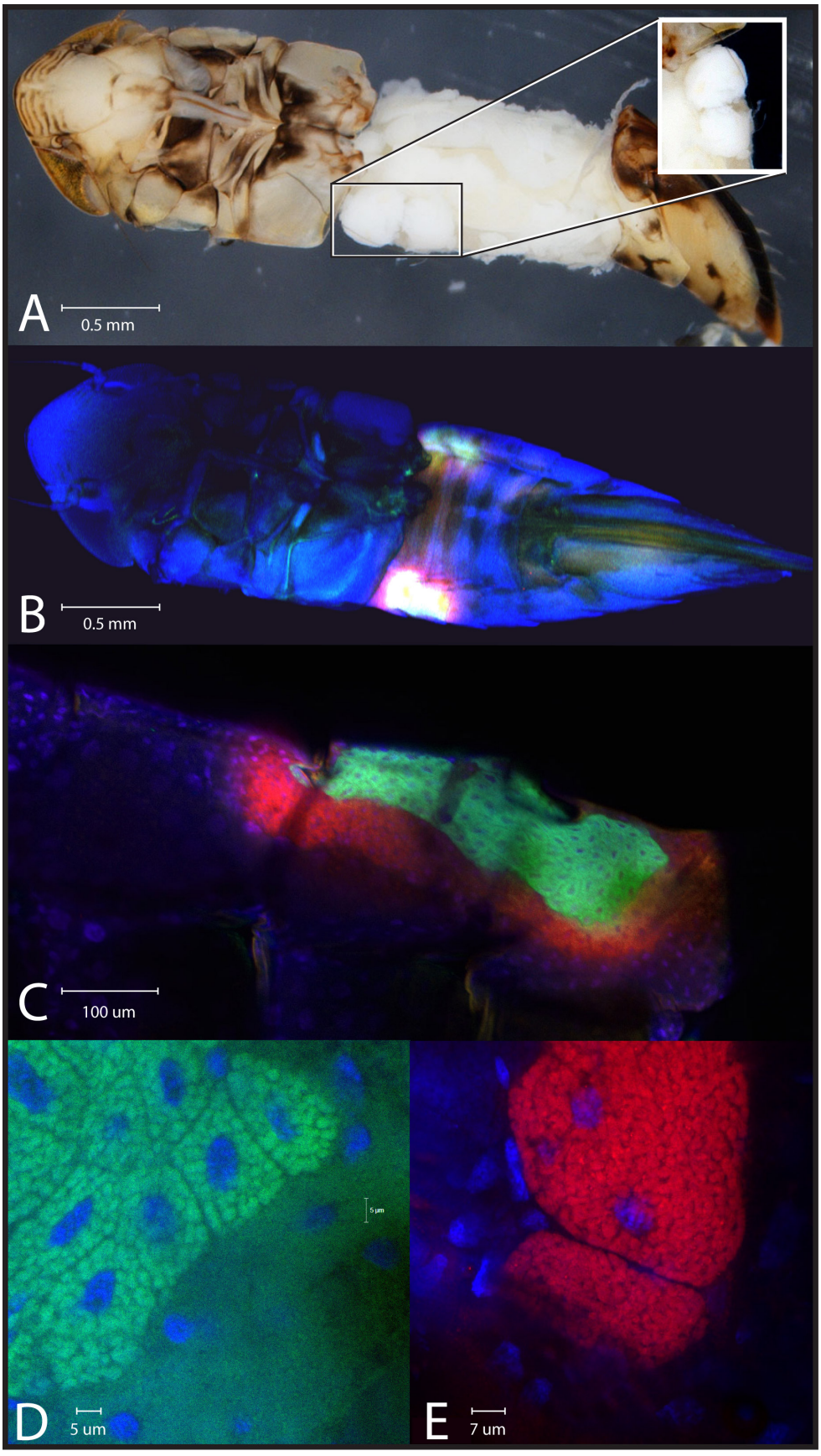
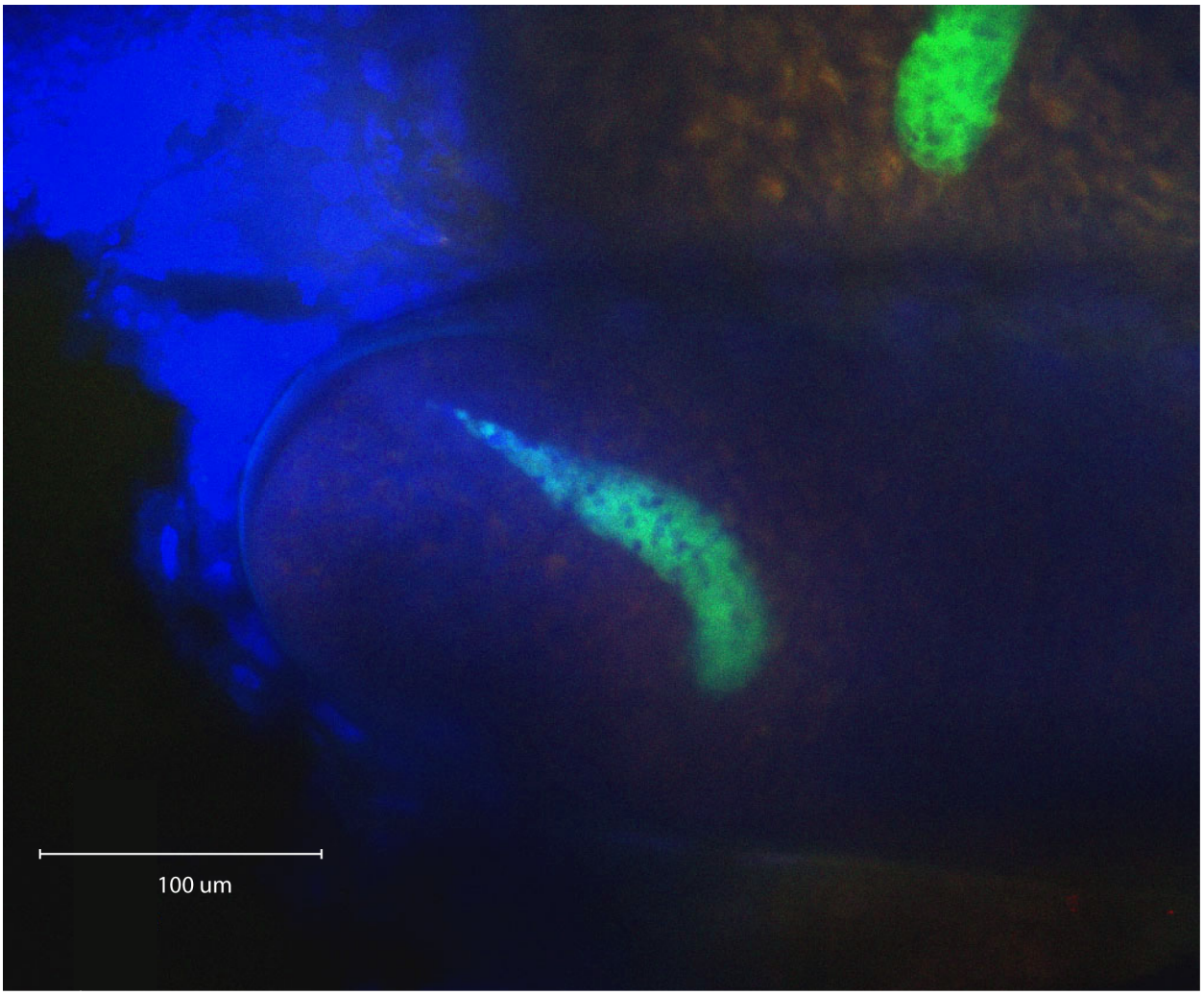
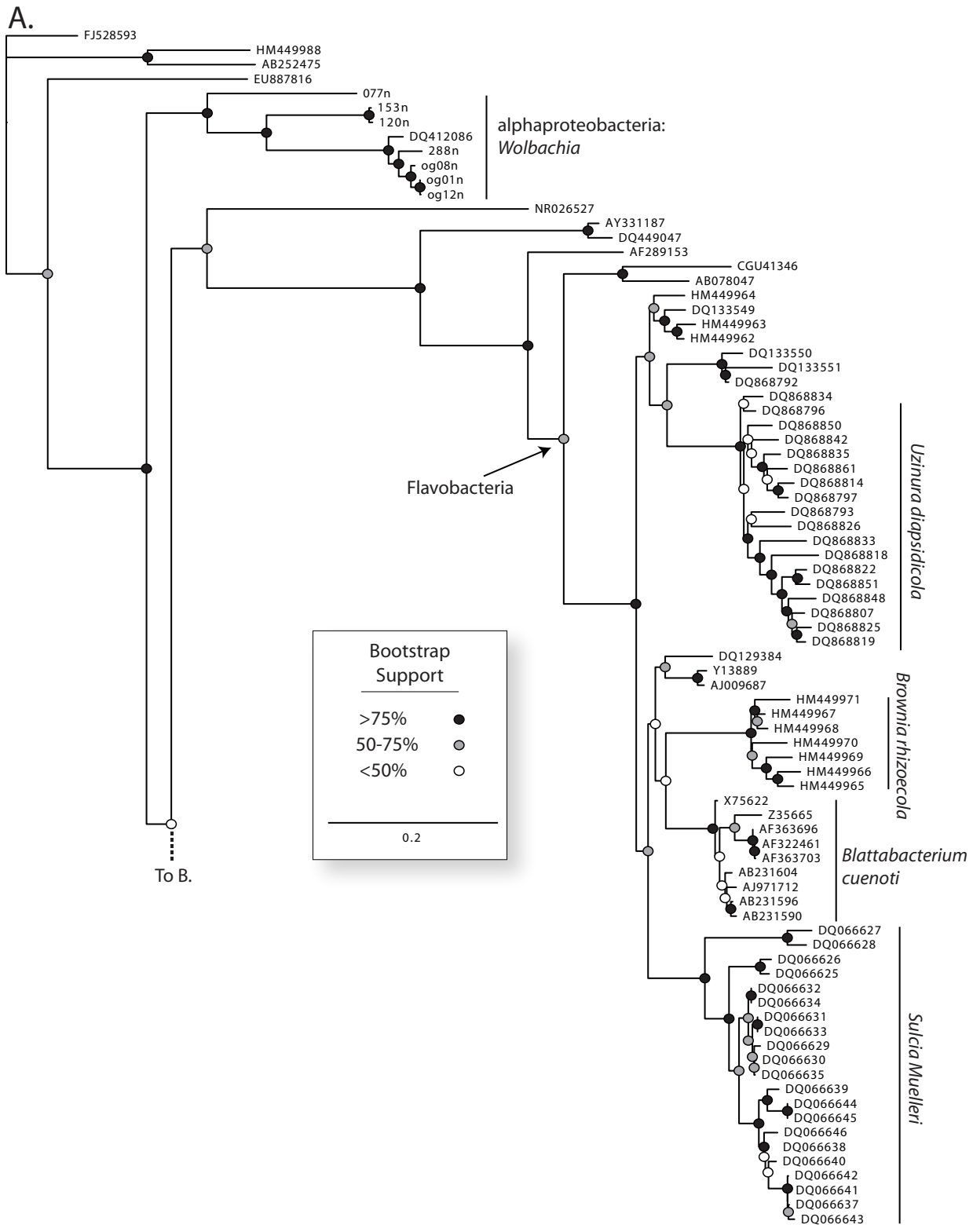

A
0.5 mm
B
0.5 mm
C
100 um
D
5 um
E
7 um

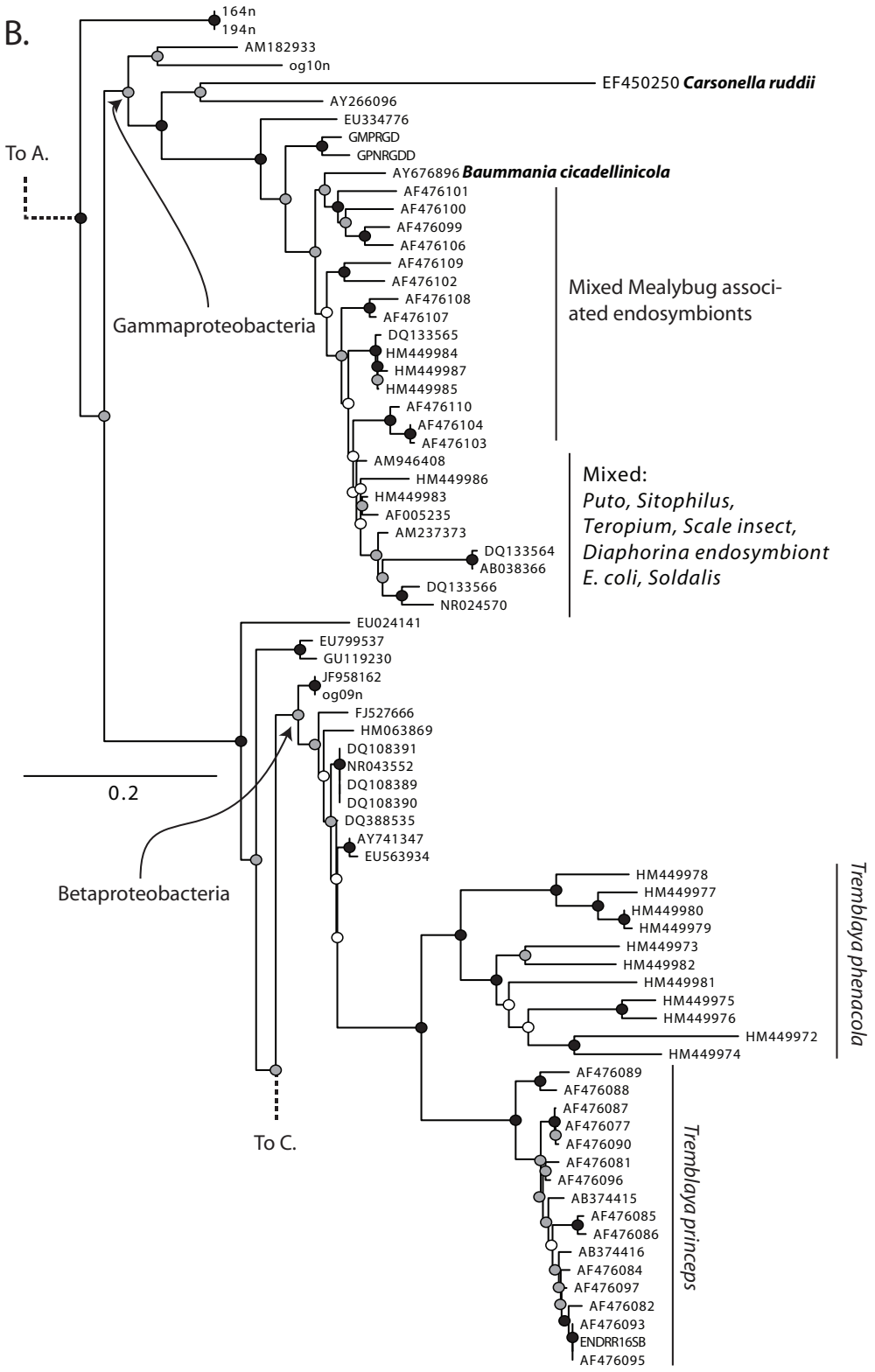
Figure 3: Fluorescent *in situ* Hybridization (FISH) imaging showing the β-proteobacterium *'Ca.* Nasuia deltocephalinicola' co-primary endosymbiont (Green) in unlaid eggs, dissected from the ovariole of *"Nesophrosyne sp.23"*. Host tissue is counter-stained with blue.



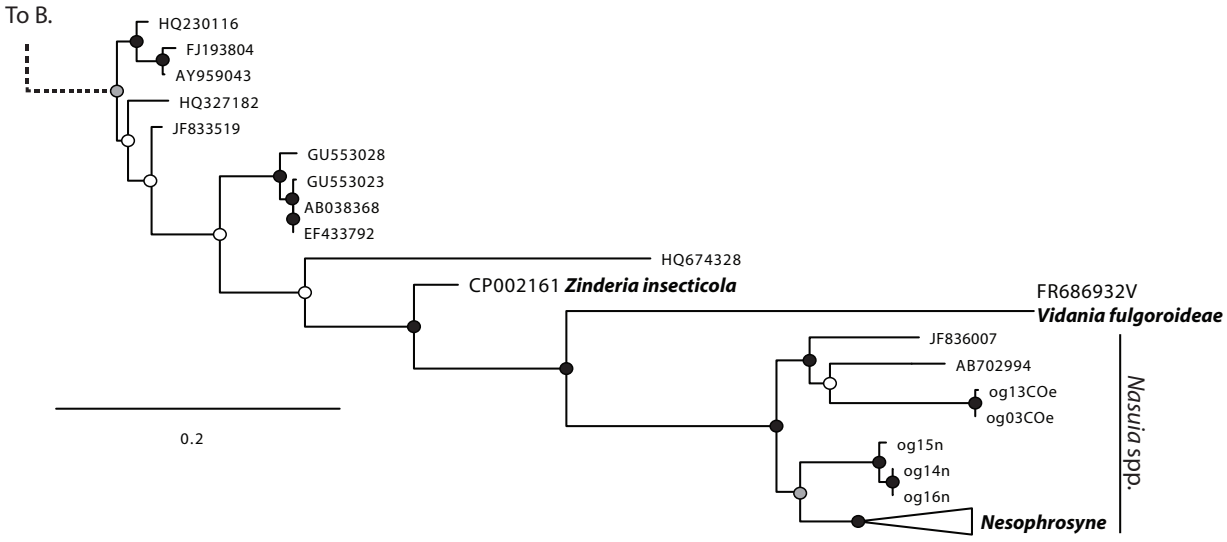
Supplementary Information

Supplementary Figure 1: Maximum likelihood phylogeny of bacterial endosymbiont lineages inferred from the 16S locus for 263 sequences. Global topology is split into three sections: A, B, and C. The major bacteria classes and known bacterial endosymbionts included in this study are labeled, corresponding to bacterial taxonomy and names (see GenBank numbers SI Table 1 for complete host taxonomic information). The tree is mid-point rooted, with nodal bootstrap support values represented as colored circles for clarity; see inset legend for corresponding values.

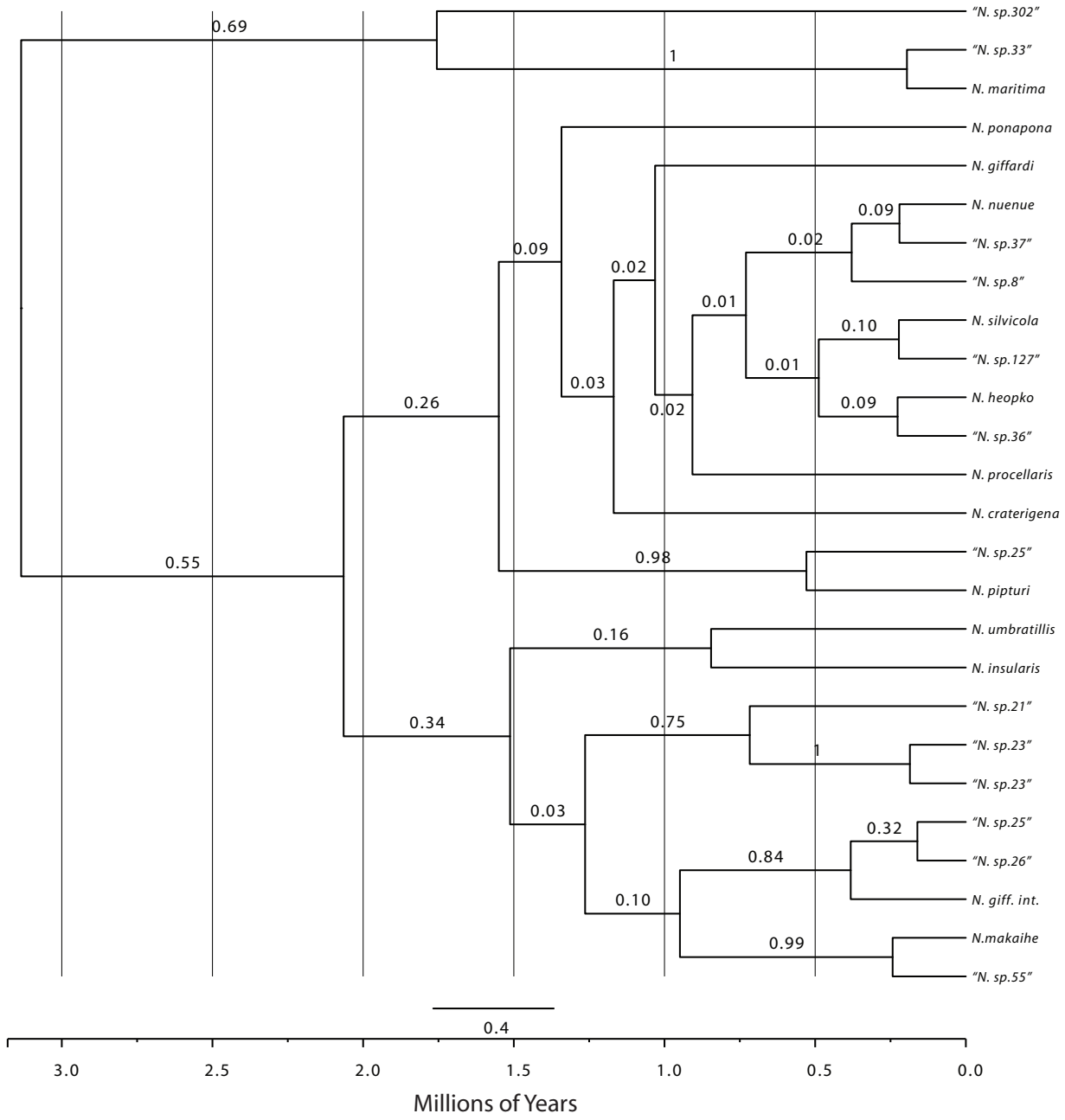

A.
FJ528593
HM449988
AB252475
EU887816
077n
153n
120n
DQ412086
288n
og08n
og01n
og12n
alphaproteobacteria:
Wolbachia
NR026527
AY331187
DQ449047
AF289153
CGU41346
AB078047
HM449964
DQ133549
HM449963
HM449962
DQ133550
DQ133551
DQ868792
DQ868834
DQ868796
DQ868850
DQ868842
DQ868835
DQ868861
DQ868814
DQ868797
DQ868793
DQ868826
DQ868833
DQ868818
DQ868822
DQ868851
DQ868848
DQ868807
DQ868825
DQ868819
Uzinura diapsidicola
Flavobacteria
Bootstrap
Support
>75%
50-75%
<50%
0.2
To B.
DQ129384
Y13889
AJ009687
HM449971
HM449967
HM449968
HM449970
HM449969
HM449966
HM449965
Brownia rhizoecola
X75622
Z35665
AF363696
AF322461
AF363703
AB231604
AJ971712
AB231596
AB231590
Blattabacterium
cuenoti
DQ066627
DQ066628
DQ066626
DQ066625
DQ066632
DQ066634
DQ066631
DQ066633
DQ066629
DQ066630
DQ066635
DQ066639
DQ066644
DQ066645
DQ066646
DQ066638
DQ066640
DQ066642
DQ066641
DQ066637
DQ066643
Sulcia Muelleri


B.
164n
194n
AM182933
og10n
EF450250 Carsonella ruddii
AY266096
EU334776
GMPRGD
GPNRGDD
AY676896 Baumannia cicadellinicola
AF476101
AF476100
AF476099
AF476106
AF476109
AF476102
AF476108
AF476107
DQ133565
HM449984
HM449987
HM449985
AF476110
AF476104
AF476103
AM946408
HM449986
HM449983
AF005235
AM237373
DQ133564
AB038366
DQ133566
NR024570
EU024141
EU799537
GU119230
JF958162
og09n
FJ527666
HM063869
DQ108391
NR043552
DQ108389
DQ108390
DQ388535
AY741347
EU563934
HM449978
HM449977
HM449980
HM449979
HM449973
HM449982
HM449981
HM449975
HM449976
HM449972
HM449974
AF476089
AF476088
AF476087
AF476077
AF476090
AF476081
AF476096
AB374415
AF476085
AF476086
AB374416
AF476084
AF476097
AF476082
AF476093
ENDRR16SB
AF476095
To A.
To C.
Gammaproteobacteria
Betaproteobacteria
0.2
Mixed Mealybug associated endosymbionts
Mixed:
Puto, Sitophilus,
Teropium, Scale insect,
Diaphorina endosymbiont
E. coli, Soldalis
Tremblaya phenacola
Tremblaya princeps

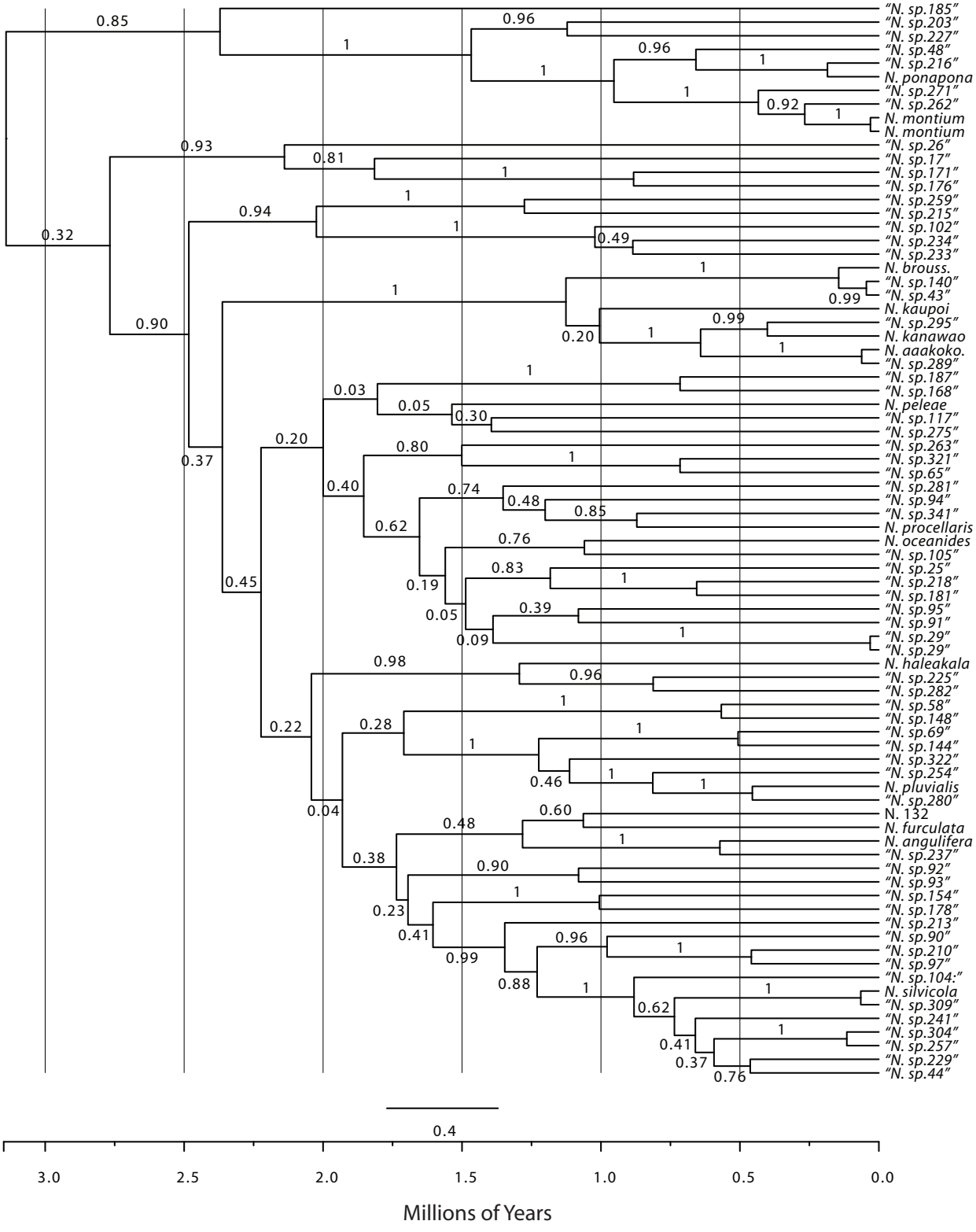
C.


To B.
HQ230116
FJ193804
AY959043
HQ327182
JF833519
GU553028
GU553023
AB038368
EF433792
HQ674328
CP002161 Zinderia insecticola
FR686932V
Vidania fulgoroideae
JF836007
AB702994
og13COe
og03COe
og15n
og14n
og16n
Nesophrosyne
Nasuia spp.
0.2

Supplementary Figure 2: Dated Bayesian phylogram for the primary bacterial endosymbiont, *Sulcia*, associated with 28 *Nesophrosyne* species (see SI Table 2 for complete taxonomic information). The tree was used to infer the absolute rates of molecular evolution presented in Table 2. Numbers above branches correspond to posterior probability support values. The bottom scale corresponds to absolute time in millions of years.


"N. sp.302"
"N. sp.33"
N. maritima
N. ponapona
N. giffardi
N. nuenue
"N. sp.37"
"N. sp.8"
N. silvicola
"N. sp.127"
N. heopko
"N. sp.36"
N. procellaris
N. craterigena
"N. sp.25"
N. pipturi
N. umbratillis
N. insularis
"N. sp.21"
"N. sp.23"
"N. sp.23"
"N. sp.25"
"N. sp.26"
N. giff. int.
N.makaihe
"N. sp.55"
0.69
1
0.09
0.02
0.09
0.02
0.01
0.10
0.01
0.03
0.09
0.02
0.26
0.55
0.98
0.16
0.34
0.75
1
0.03
0.32
0.84
0.10
0.99
0.4
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Millions of Years

Supplementary Figure 3: Dated Bayesian phylogram for the co-primary β-proteobacterium, *Nasuia*, associated with 79 *Nesophrosyne* species (see SI Table 2 for complete taxonomic information). The tree was used to infer the absolute rates of molecular evolution presented in Table 2. Numbers above branches correspond to posterior probability support values. Scale corresponds to absolute time in millions of years.


"N. sp.185"
"N. sp.203"
"N. sp.227"
"N. sp.48"
"N. sp.216"
N. ponapona
"N. sp.271"
"N. sp.262"
N. montium
N. montium
"N. sp.26"
"N. sp.17"
"N. sp.171"
"N. sp.176"
"N. sp.259"
"N. sp.215"
"N. sp.102"
"N. sp.234"
"N. sp.233"
N. brouss.
"N. sp.140"
"N. sp.43"
N. kaupoi
"N. sp.295"
N. kanawao
N. aaakoko.
"N. sp.289"
"N. sp.187"
"N. sp.168"
N. peleae
"N. sp.117"
"N. sp.275"
"N. sp.263"
"N. sp.321"
"N. sp.65"
"N. sp.281"
"N. sp.94"
"N. sp.341"
N. procellaris
N. oceanides
"N. sp.105"
"N. sp.25"
"N. sp.218"
"N. sp.181"
"N. sp.95"
"N. sp.91"
"N. sp.29"
"N. sp.29"
N. haleakala
"N. sp.225"
"N. sp.282"
"N. sp.58"
"N. sp.148"
"N. sp.69"
"N. sp.144"
"N. sp.322"
"N. sp.254"
N. pluvialis
"N. sp.280"
N. 132
N. furculata
N. angulifera
"N. sp.237"
"N. sp.92"
"N. sp.93"
"N. sp.154"
"N. sp.178"
"N. sp.213"
"N. sp.90"
"N. sp.210"
"N. sp.97"
"N. sp.104:"
N. silvicola
"N. sp.309"
"N. sp.241"
"N. sp.304"
"N. sp.257"
"N. sp.229"
"N. sp.44"
0.4
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Millions of Years

Supplementary Information

Supplementary Table 1: Bacterial taxonomic sampling, including endosymbiont status (Env or Endo), related host taxonomic inoformation, and corresponding citations.

GenBank / Study #	Phylum	Class	Bacterium name	Env or Endo	Host Order	Host Family	*Host Genus*	*Host species*	Citations
AY266096	Proteobacteria	Gammaproteobacteria	Protiera aleyrodidarum	Endo	Hemiptera	Aleyrodidae	*Aleurodicus*	*dispersus*	Thao and Baumann 2004
AY331187	Bacteriodetes	Sphingobacteria	Cardinium hertigii	Endo	Hymenoptera	Aphelinidae	*Encarsia*	*hispida*	Zchori-Fein et al 2004
DQ066630	Bacteriodetes	Flavobacteria	Sulcia muelleri	Endo	Hemiptera	Aphoridae	*Microsargane*	*sp.*	Moran et al 2005
DQ066629	Bacteriodetes	Flavobacteria	Sulcia muelleri	Endo	Hemiptera	Aphoridae	*Aphrophora*	*quadtinotata*	Moran et al 2005
X75622	Bacteriodetes	Flavobacteria	Blattabacterium sp.	Endo	Blattaria	Blaberidae	*N.*	*cinerea*	Bandi et al. 1995
AB231604	Bacteriodetes	Flavobacteria	Blattabacterium sp.	Endo	Blattodea	Blaberidae	*Panesthia*	*angustipennis*	Maekawa et al 2005
AB231596	Bacteriodetes	Flavobacteria	Blattabacterium sp.	Endo	Blattodea	Blaberidae	*Salganea*	*taiwanensis*	Maekawa et al 2005
AB231590	Bacteriodetes	Flavobacteria	Blattabacterium sp.	Endo	Blattodea	Blaberidae	*Salganea*	*esakii*	Maekawa et al 2005
AJ971712	Bacteriodetes	Flavobacteria	Blattabacterium sp.	Endo	Blattaria	Blattellidae	*Blatella*	*germanica*	Neef et al 2005
AM946408	Undet			Endo	Coleoptera	Cerambycidae	*Tetropium*	*castaneum*	Gruenwald 2008 unpub
DQ066635	Bacteriodetes	Flavobacteria	Sulcia muelleri	Endo	Hemiptera	Cercopidae	*Mahanarva*	*costaricensis*	Moran et al 2005
DQ066625	Bacteriodetes	Flavobacteria	Sulcia muelleri	Endo	Hemiptera	Cicadedae	*Magicicada*	*septendecim*	Moran et al 2005
DQ066626	Bacteriodetes	Flavobacteria	Sulcia muelleri	Endo	Hemiptera	Cicadedae	*Diceroprocta*	*apache*	Moran et al 2005
DQ066645	Bacteriodetes	Flavobacteria	Sulcia muelleri	Endo	Hemiptera	Cicadellidae	*Excultanus*	*nr.excultus*	Moran et al 2005
DQ066644	Bacteriodetes	Flavobacteria	Sulcia muelleri	Endo	Hemiptera	Cicadellidae	*Excultanus*	*sp.*	Moran et al 2005
DQ066639	Bacteriodetes	Flavobacteria	Sulcia muelleri	Endo	Hemiptera	Cicadellidae	*Acinopterus*	*sp.*	Moran et al 2005
DQ066640	Bacteriodetes	Flavobacteria	Sulcia muelleri	Endo	Hemiptera	Cicadellidae	*Jikradia*	*olitoria*	Moran et al 2005
DQ066646	Bacteriodetes	Flavobacteria	Sulcia muelleri	Endo	Hemiptera	Cicadellidae	*Homalodisca*	*coagulata*	Moran et al 2005
DQ066638	Bacteriodetes	Flavobacteria	Sulcia muelleri	Endo	Hemiptera	Cicadellidae	*Hamana*	*dictatoria*	Moran et al 2005
AY676896	Proteobacteria	Gammaproteobacteria	Baumannia cicadellinicola	Endo	Hemiptera	Cicadellidae	*Paromenia*	*isabellina*	Takiya et al 2006
JF836007	Proteobacteria	Betaproteobacteria	Nasuia deltocephalinicola	Endo	Hemiptera	Cicadellidae	*Matsumuratettix*	*hiroglyphicus*	Wangkeeree et al. 2011
og01COe1	Proteobacteria	Alphaproteobacteria	Wolbachia	Endo	Hemiptera	Cicadellidae	*Nesophyla*	*variata*	This study
og03COe	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Deltocephalus*	*sp.*	This study
og08COe	Proteobacteria	Alphaproteobacteria	Wolbachia	Endo	Hemiptera	Cicadellidae	*Opsius*	*stactogalus*	This study
og09COe	Undet			Endo	Hemiptera	Cicadellidae	*Plagotettix*	*cyclops*	This study
og10COe	Undet			Endo	Hemiptera	Cicadellidae	*Lampridius*	*sp.*	This study
og12COe	Proteobacteria	Alphaproteobacteria	Wolbachia	Endo	Hemiptera	Cicadellidae	*Opsianus*	*sp.*	This study
og13COe	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Deltocephalus*	*sp.*	This study
og14COe	Proteobacteria	Betaproteobacteria	Nasuia	Endo	Hemiptera	Cicadellidae	*Orosius*	*orientalis*	This study

			zimmermani						
og15COe	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Orosius*	*sp.*	This study
og16COe	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Orosius*	*orientalis*	This study
007n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*N. sivilcola*	This study
103CO16s	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*N. procellaris*	This study
212n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*N. ponapona*	This study
290n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*N. pluvialis*	This study
109n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*N. broussaisiai*	This study
188n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*N. peleae*	This study
291n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*N. oceanides*	This study
088CO16s	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*N. notatula*	This study
308n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*N. montivaga*	This study
279n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*N. montium*	This study
074n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*N. mabae*	This study
113n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*N. kaupoi*	This study
123COe16	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*N. kanawao*	This study
050n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*N. haleakala*	This study
324n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*N. furculata*	This study
077BetaR	Proteobacteria	Alphaproteobacteria	Wolbachia	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*N. eburneola*	This study
059n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*N. anguilifera*	This study
124n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*N. aakokohaikea*	This study
097n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.97"*	This study
095n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.95"*	This study
094n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.94"*	This study
093n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.93"*	This study
092n	Proteobacteria	Betaproteobacteria	Nasuia	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.92"*	This study

			zimmermani						
091CO16s	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.91"*	This study
090n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.90"*	This study
069n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.69"*	This study
065n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.65"*	This study
058n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.58"*	This study
048n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.48"*	This study
044n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.44"*	This study
190COe16	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.341"*	This study
322n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.322"*	This study
321n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.321"*	This study
318n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.318"*	This study
316n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.316"*	This study
076n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.309"*	This study
297n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.304"*	This study
295n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.295"*	This study
163n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.29"*	This study
289n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.289"*	This study
282n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.282"*	This study
281n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.281"*	This study
280n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.280"*	This study
275n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.275"*	This study
271n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.271"*	This study
263n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.263"*	This study
262n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.262"*	This study

026n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.26"*	This study
259n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.259"*	This study
257n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.257"*	This study
254n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.254"*	This study
025n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.25"*	This study
245n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.245"*	This study
241n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.241"*	This study
237n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.237"*	This study
234n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.234"*	This study
233n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.233"*	This study
229n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.229"*	This study
227n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.227"*	This study
225n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.225"*	This study
218n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.218"*	This study
206n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.216"*	This study
215n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.215"*	This study
213n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.213"*	This study
210n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.210"*	This study
202n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.203"*	This study
187n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.187"*	This study
185n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.186"*	This study
184n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.184"*	This study
160COe16	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.181"*	This study
178n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.178"*	This study
176n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.176"*	This study

175n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.175"*	This study
171n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.171"*	This study
017n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.17"*	This study
168n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.168"*	This study
154n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.154"*	This study
153n	Proteobacteria	Alphaproteobacteria	Wolbachia	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.153"*	This study
148n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.148"*	This study
144n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.144"*	This study
140COe16	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.140"*	This study
132n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.132"*	This study
288n	Proteobacteria	Alphaproteobacteria	Wolbachia	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.125"*	This study
135n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.117"*	This study
105n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.105"*	This study
104n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.104"*	This study
102n	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.102"*	This study
043CO16s	Proteobacteria	Betaproteobacteria	Nasuia zimmermani	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.43"*	This study
AB702994	Proteobacteria	Betaproteobacteria	Nasuia deltocephanilicola	Endo	Hemiptera	Cicadellidae	*Nephotettix*	*cinticeps*	Noda et al 2012
120n	Proteobacteria	Alphaproteobacteria	Wolbachia	Endo	Hemiptera	Cicadellidae	*Nesophrosyne*	*"N. sp.120"*	this study
FR686932V	Proteobacteria	Betaproteobacteria	Vidania folgoroideae	Endo	Hemiptera	Cixiidae	*Hyalesthes*	*obsoletus*	Gonella ey al 2011
DQ066632	Bacteriodetes	Flavobacteria	Sulcia muelleri	Endo	Hemiptera	Clastopteridae	*Clastopera*	*arizonana*	Moran et al 2005
DQ066634	Bacteriodetes	Flavobacteria	Sulcia muelleri	Endo	Hemiptera	Clastopteridae	*Clastopera*	*obtusa*	Moran et al 2005
CP002161	Proteobacteria	Betaproteobacteria	Zinderia insecticola	Endo	Hemiptera	Clastopteridae	*Clastoptera*	*arizonana*	McCutcheon et al 2010
DQ133566	Proteobacteria	Undet		Endo	Hemiptera	Coccidae	*Saissetia*	*oleae*	Gruwell et al 2010
DQ133564	Proteobacteria	Undet		Endo	Hemiptera	Coccidae	*Coccus*	*hesperidum*	Gruwell unpub 2005
AJ009687	Bacteriodetes	Flavobacteria	Flavobacterium sp.	Endo	Coleoptera	Coccinellidae	*Adonia*	*variegata*	Hurst et al 1999
Y13889	Bacteriodetes	Flavobacteria		Endo	Coleoptera	Coccinellidae	*Coleomegilla*	*maculata*	Hurst et al. 1997
HM449973	Proteobacteria	Betaproteobacteria	Candudatus Tremblaya phenacola	Endo	Hemiptera	Coccooidea	*Mirococcus*	*sp.*	Gruwell et al 2010

HM449981	Proteobacteria	Betaproteobacteria	Candudatus Tremblaya phenacola	Endo	Hemiptera	Coccooidea	*Heliococcus*	*clemente*	Gruwell et al 2010
AF363696	Bacteriodetes	Flavobacteria	Blattabacterium punctulatus	Endo	Blattodea	Cryptocercidae	*Cryptocercus*	*sp.*	Clark and Kambhampati 2003
AF363703	Bacteriodetes	Flavobacteria	Blattabacterium punctulatus	Endo	Blattodea	Cryptocercidae	*Cryptocercus*		Clark and Kambhampati 2003
AF322461	Bacteriodetes	Flavobacteria	Blattabacterium sp.	Endo	Blattodea	Cryptocercidae	*Cryptocercus*	*darwini*	Clark et al. 2001
AF005235	Proteobacteria	Gammaproteobacteria		Endo	Coleoptera	Curculionidae	*Sitophilius*	*oryzae*	Heddi et al 1998
DQ868850	Bacteriodetes	Flavobacteria		Endo	Hemiptera	Diaspididae	*Pseudaonidia*	*trilobitiformis*	Gruwell et al 2007
DQ868825	Bacteriodetes	Flavobacteria	Uzinura diaspidicola	Endo	Hemiptera	Diaspididae	*Duplachionaspis*	*divergens*	Gruwell et al 2010
DQ868851	Bacteriodetes	Flavobacteria	undet	Endo	Hemiptera	Diaspididae	Pseudaulacaspis	*cockerelli*	Gruwell et al 2007
DQ868833	Bacteriodetes	Flavobacteria	undet	Endo	Hemiptera	Diaspididae	*Lepidosaphes*	*pinnaeformis*	Gruwell et al 2007
DQ868818	Bacteriodetes	Flavobacteria	undet	Endo	Hemiptera	Diaspididae	*Diaspis*	*manzanitae*	Gruwell et al 2007
DQ868826	Bacteriodetes	Flavobacteria	Uzinura diaspidicola	Endo	Hemiptera	Diaspididae	*Howardia*	*biclavis*	Gruwell et al 2007
DQ868842	Bacteriodetes	Flavobacteria	Uzinura diaspidicola	Endo	Hemiptera	Diaspididae	*Parlatoria*	*pergandei*	Gruwell et al 2007
DQ868835	Bacteriodetes	Flavobacteria	Uzinura diaspidicola	Endo	Hemiptera	Diaspididae	*Melanaspis*	*bromeliae*	Gruwell et al 2007
DQ868797	Bacteriodetes	Flavobacteria	Uzinura diaspidicola	Endo	Hemiptera	Diaspididae	*Dynaspidiotus*	*degeneratus*	Gruwell et al 2007
DQ868822	Bacteriodetes	Flavobacteria	Uzinura diaspidicola	Endo	Hemiptera	Diaspididae	*Fiorinia*	*euryae*	Gruwell et al 2007
DQ868848	Bacteriodetes	Flavobacteria	Uzinura diaspidicola	Endo	Hemiptera	Diaspididae	*Pinnaspis*	*hibisci*	Gruwell et al 2007
DQ868807	Bacteriodetes	Flavobacteria	Uzinura diaspidicola	Endo	Hemiptera	Diaspididae	*Chionaspis*	*pinifoliae*	Gruwell et al 2007
DQ868819	Bacteriodetes	Flavobacteria	Uzinura diaspidicola	Endo	Hemiptera	Diaspididae	*Duplachionaspis*	*noaeae*	Gruwell et al 2007
DQ868814	Bacteriodetes	Flavobacteria	Uzinura diaspidicola	Endo	Hemiptera	Diaspididae	*Hemiberlesia*	*lataniae*	Gruwell et al 2007
DQ868796	Bacteriodetes	Flavobacteria	Uzinura diaspidicola	Endo	Hemiptera	Diaspididae	*Leucaspis*	*ohakunensis*	Gruwell et al 2007
DQ868834	Bacteriodetes	Flavobacteria	Uzinura diaspidicola	Endo	Hemiptera	Diaspididae	*Maskellia*	*globosa*	Gruwell et al 2007
DQ868861	Bacteriodetes	Flavobacteria	Uzinura diaspidicola	Endo	Hemiptera	Diaspididae	*Aspidiotus*	*nerii*	Gruwell et al 2007
DQ868793	Bacteriodetes	Flavobacteria	Uzinura diaspidicola	Endo	Hemiptera	Diaspididae	*Prodigiaspis*	*sp.*	Gruwell et al 2007
DQ868792	Bacteriodetes	Flavobacteria		Endo	Hemiptera	Diaspididae	*Icerya*	*brasiliensis*	Gruwell et al 2007
DQ133551	Bacteriodetes	Undet		Endo	Hemiptera	Diaspididae	*Icerya*	*brasiliensis*	Gruwell unpub 2005
DQ133550	Bacteriodetes	Undet		Endo	Hemiptera	Diaspididae	*Icerya*	*purchasi*	Gruwell unpub 2005
DQ066627	Bacteriodetes	Flavobacteria	Sulcia muelleri	Endo	Hemiptera	Dictyopharida	*Scolops*	*sp.*	Moran et al 2005

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DQ412086	Proteobacteria	Alphaproteobacteria	Wolbachia	Endo	Diptera	Drosophilidae	*Drosophila*	*willistoni*	Matteos et al. 2006
AB252475	Tenericutes	Mollicutes	Spiroplasma	Endo	Diptera	Drosophilidae	*Drosophila*	*hydei*	Kageyama et al 2006
DQ133549	Bacteriodetes	undet		Endo	Hemiptera	Eriococcidae	*Cryptococcus*	*ulmi*	Gruwell et al 2005
FJ527666	Proteobacteria	Betaproteobacteria	Burkholderia sp.	Endo (plant)	Gabales	Fabaceae	*Lespedeza*	*floribunda*	Peng and Jiao 2008 unpub
DQ066628	Bacteriodetes	Flavobacteria	Sulcia muelleri	Endo	Hemiptera	Fulgoridae	*Poblicia*	*fuliginosa*	Moran et al 2005
GMPRGD	Proteobacteria	Gammaproteobacteria	Wigglesworthia glossinidia	Endo	Diptera	Glossinidae	*Glossina*	*morsitans*	Aksoy 1995
GPNRGDD	Proteobacteria	Gammaproteobacteria	Wigglesworthia glossinidia	Endo	Diptera	Glossinidae	*Glossina*	*palpalis*	Aksoy 1995
EU334776	Proteobacteria	Gammaproteobacteria	Buchnera aphidicola	Endo	Hemiptera	Lachnidae	*Cinara*	*pilicornis*	Lamelas et al. 2008
DQ066633	Bacteriodetes	Flavobacteria	Sulcia muelleri	Endo	Hemiptera	Machaerotidae	*Chaetophyes*	*vicina*	Moran et al 2005
DQ066631	Bacteriodetes	Flavobacteria	Sulcia muelleri	Endo	Hemiptera	Machaerotidae	*Hindoloides*	*bipunctulatus*	Moran et al 2005
Z35665	Bacteriodetes	Flavobacteria	Blattabacterium sp.	Endo	Isoptera	Mastotermitidae	*Mastotermes*	*dariniensis*	Bandi et al 1995
DQ066641	Bacteriodetes	Flavobacteria	Sulcia muelleri	Endo	Hemiptera	Membracidae	*Publilia*	*modesta*	Moran et al 2005
DQ066637	Bacteriodetes	Flavobacteria	Sulcia muelleri	Endo	Hemiptera	Membracidae	*Spissistilus*	*festinus*	Moran et al 2005
DQ066642	Bacteriodetes	Flavobacteria	Sulcia muelleri	Endo	Hemiptera	Membracidae	*Phylya*	*ferruginosa*	Moran et al 2005
DQ066643	Bacteriodetes	Flavobacteria	Sulcia muelleri	Endo	Hemiptera	Membracidae	*Platycotis*	*vittata*	Moran et al 2005
AM182933	Proteobacteria	Gammaproteobacteria	Pseudomonas viridiflava	Endo (Plant)	Lamiales	Plantaginaceae	*Hebe*	*spp.*	Gonzalez and Rodicio unpub 2006
HM449988	Uncultured	Uncultured		Endo	Hemiptera	Pseudococcidae	Puto	*albicans*	Gruwell et al 2010
HM449970	Bacteriodetes	Flavobacteria	Cadidatus Brownia	Endo	Hemiptera	Pseudococcidae	*Neochavesia*	*caldasiae*	Gruwell et al 2010
HM449978	Proteobacteria	Betaproteobacteria	Tremblaya	Endo	Hemiptera	Pseudococcidae	*Phenacoccus*	*madeirensis*	Gruwell et al 2007
HM449964	Undetermined			Endo	Hemiptera	Pseudococcidae	*Rastrococcus*	*invadens*	Gruwell et al 2010
HM449963	Undetermined			Endo	Hemiptera	Pseudococcidae	*Rastrococcus*	*invadens*	Gruwell et al 2010
HM449962	Undetermined			Endo	Hemiptera	Pseudococcidae	*Rastrococcus*	*asteliae*	Gruwell et al 2010
HM449987	Undetermined			Endo	Hemiptera	Pseudococcidae	*Puto*	*yuccae*	Gruwell et al 2010
HM449986	Undetermined			Endo	Hemiptera	Pseudococcidae	*Puto*	*albicans*	Gruwell et al 2010
HM449967	Bacteriodetes	Flavobacteria	Brownia rhizoecola	Endo	Hemiptera	Pseudococcidae	*Rhizoecus*	*hibisci*	Gruwell et al 2010
HM449968	Bacteriodetes	Flavobacteria	Brownia rhizoecola	Endo	Hemiptera	Pseudococcidae	*Geococcus*	*coffeae*	Gruwell et al 2010
HM449971	Bacteriodetes	Flavobacteria	Brownia rhizoecola	Endo	Hemiptera	Pseudococcidae	*Rhizoecus*	*falcifer*	Gruwell et al 2010

HM449969	Bacteriodetes	Flavobacteria	Brownia rhizoecola	Endo	Hemiptera	Pseudococcidae	*Rhizoecus*	*gracilis*	Gruwell et al 2010
HM449965	Bacteriodetes	Flavobacteria	Brownia rhizoecola	Endo	Hemiptera	Pseudococcidae	*Rhizoecus*	*floridanus*	Gruwell et al 2010
HM449966	Bacteriodetes	Flavobacteria	Brownia rhizoecola	Endo	Hemiptera	Pseudococcidae	*Rhizoecus*	*cacticans*	Gruwell et al 2010
AF476100	Proteobacteria	Gammaproteobacteria	Undet mealy second	Endo	Hemiptera	Pseudococcidae	*Amonostheriu m*	*lichtensiodes*	Thao et al 2002
AF476101	Proteobacteria	Gammaproteobacteria	Undet mealy second	Endo	Hemiptera	Pseudococcidae	*Antonina*	*pertiosa*	Thao et al 2002
AF476099	Proteobacteria	Gammaproteobacteria		Endo	Hemiptera	Pseudococcidae	*Australicoccus*	*grevilleae*	Thao et al 2002
AF476106	Proteobacteria	Gammaproteobacteria	Undet mealy second	Endo	Hemiptera	Pseudococcidae	*Melanococcus*	*albizziae*	Thao et al 2002
AF476109	Proteobacteria	Gammaproteobacteria	Undet mealy second	Endo	Hemiptera	Pseudococcidae	*Paracoccus*	*nothofagicola*	Thao et al 2002
HM449974	Proteobacteria	Betaproteobacteria	Candudatus Tremblaya phenacola	Endo	Hemiptera	Pseudococcidae	*Peliococcus*	*turanicus*	Gruwell et al 2010
AF476107	Proteobacteria	Gammaproteobacteria	Undet mealy second	Endo	Hemiptera	Pseudococcidae	*Planococcus*	*citri*	Thao et al 2002
AF476104	Proteobacteria	Gammaproteobacteria	Undet mealy second	Endo	Hemiptera	Pseudococcidae	*Dysmicoccus*	*neobervipes*	Thao et al 2002
DQ133565	Proteobacteria	Undet		Endo	Hemiptera	Pseudococcidae	*Puto*	*sp.*	Gruwell et al 2005
AF476108	Proteobacteria	Gammaproteobacteria	Undet mealy second	Endo	Hemiptera	Pseudococcidae	*Planococcus*	*ficus*	Thao et al 2002
AF476102	Proteobacteria	Gammaproteobacteria	Undet mealy second	Endo	Hemiptera	Pseudococcidae	*Cyphonococcu s*	*alpinus*	Thao et al 2002
AF476103	Proteobacteria	Gammaproteobacteria	Undet mealy second	Endo	Hemiptera	Pseudococcidae	*Dysmicoccus*	*bervipes*	Thao et al 2002
AF476110	Proteobacteria	Gammaproteobacteria	Undet mealy second	Endo	Hemiptera	Pseudococcidae	*Vryburgia*	*amaryllidis*	Thao et al 2002
HM449975	Proteobacteria	Betaproteobacteria	Tremblaya phenacola	Endo	Hemiptera	Pseudococcidae	*Phenacoccus*	*sp.*	Gruwell et al 2010
HM449976	Proteobacteria	Betaproteobacteria	Tremblaya phenacola	Endo	Hemiptera	Pseudococcidae	*Heterococcus*	*nudus*	Gruwell et al 2010
HM449980	Proteobacteria	Betaproteobacteria	Tremblaya phenacola	Endo	Hemiptera	Pseudococcidae	*Phenacoccus*	*solani*	Gruwell et al 2010
HM449979	Proteobacteria	Betaproteobacteria	Tremblaya phenacola	Endo	Hemiptera	Pseudococcidae	*Phenacoccus*	*solani*	Gruwell et al 2010
AF476084	Proteobacteria	Betaproteobacteria	Tremblaya princeps	Endo	Hemiptera	Pseudococcidae	*Erium*	*globosum*	Thao et al 2002
AB374416	Proteobacteria	Betaproteobacteria	Tremblaya princeps	Endo	Hemiptera	Pseudococcidae	*Pseudococcus*	*comstocki*	Kono et al 2008
AF476097	Proteobacteria	Betaproteobacteria	Tremblaya princeps	Endo	Hemiptera	Pseudococcidae	*Vryburgia*	*amaryllidis*	Thao et al 2002
AF476082	Proteobacteria	Betaproteobacteria	Tremblaya princeps	Endo	Hemiptera	Pseudococcidae	*Dysmicoccus*	*bervipes*	Thao et al 2002

AF476093	Proteobacteria	Betaproteobacteria	Tremblaya princeps	Endo	Hemiptera	Pseudococcidae	*Pseudococcus*	*longispinus*	Thao et al 2002
AF476095	Proteobacteria	Betaproteobacteria	Tremblaya princeps	Endo	Hemiptera	Pseudococcidae	*Pseudococcus*	*viburni*	Thao et al 2002
ENDRR16SB	Undet			Endo	Hemiptera	Pseudococcidae	*Pseudococcus*	*longispinus*	Munson et al 1992
AB374415	Proteobacteria	Betaproteobacteria	Tremblaya princeps	Endo	Hemiptera	Pseudococcidae	*Planococcus*	*kraunhiae*	Kono et al 208
AF476090	Proteobacteria	Betaproteobacteria	Tremblaya princeps	Endo	Hemiptera	Pseudococcidae	*Nipaecoccus*	*exocarpi*	Thao et al 2002
AF476087	Proteobacteria	Betaproteobacteria	Tremblaya princeps	Endo	Hemiptera	Pseudococcidae	*Melanococcus*	*albizziae*	Thao et al 2002
AF476077	Proteobacteria	Betaproteobacteria	Tremblaya princeps	Endo	Hemiptera	Pseudococcidae	*Australicoccus*	*grevilleae*	Thao et al 2002
HM449977	Proteobacteria	Betaproteobacteria	Tremblaya phenacola	Endo	Hemiptera	Pseudococcidae	*Phenacoccus*	*manihoti*	Gruwell et al 2010
AF476085	Proteobacteria	Betaproteobacteria	Tremblaya princeps	Endo	Hemiptera	Pseudococcidae	*Ferrisia*	*malvastra*	Thao et al 2002
AF476086	Proteobacteria	Betaproteobacteria	Tremblaya princeps	Endo	Hemiptera	Pseudococcidae	*Ferrisia*	*sp.*	Thao et al 2002
AF476096	Proteobacteria	Betaproteobacteria	Tremblaya princeps	Endo	Hemiptera	Pseudococcidae	*Sarococcus*	*comis*	Thao et al 2002
HM449972	Proteobacteria	Betaproteobacteria	Tremblaya phenacola	Endo	Hemiptera	Pseudococcidae	*Osyacanthus*	*sp.*	Gruwell et al 2010
AF476081	Proteobacteria	Betaproteobacteria	Tremblaya princeps	Endo	Hemiptera	Pseudococcidae	*Cyphonococcus*	*alpinus*	Thao et al 2002
AF476089	Proteobacteria	Betaproteobacteria	Tremblaya princeps	Endo	Hemiptera	Pseudococcidae	*Maconellicoccus*	*hirsutus*	Thao et al 2002
HM449982	Proteobacteria	Betaproteobacteria	Tremblaya phenacola	Endo	Hemiptera	Pseudococcidae	*Phenacoccus*	*aceris*	Thao et al 2002
HM449985	Undet			Endo	Hemiptera	Pseudococcidae	*Puto*	*albicans*	Gruwell et al 2010
HM449984	Undet			Endo	Hemiptera	Pseudococcidae	*Puto*	*albicans*	Gruwell et al 2010
AF476088	Proteobacteria	Betaproteobacteria	Tremblaya princeps	Endo	Hemiptera	Pseudococcidae	*Maconellicoccus*	*australiensis*	Thao et al 2002
EF433792	Proteobacteria	Betaproteobacteria		Endo	Hemiptera	Psyllidae	*Diaphorina*	*citri*	Meyer and Hoy 2008
AB038366	Proteobacteria	Gammaproteobacteria	Arsenophonus sp.	Endo	Hemiptera	Psyllidae	*Diaphornia*	*citri*	Subandiyah et al 2000
GU553028	Undet			Endo	Hemiptera	Psyllidae	*Diaphorina*	*citri*	Tian et al. 2010
EF450250	Proteobacteria	Gammaproteobacteria	Carsonella rudi	Endo	Hemiptera	Psyllidae	*Diaphorina*	*citri*	Meyer and Hoy 2008
GU553023	Undet			Endo	Hemiptera	Psyllidae	*Diaphorina*	*citri*	Tian et al. 2010 unpub
AB038368	Proteobacteria	Betaproteobacteria		Endo	Hemiptera	Psyllidae	*Diaphorina*	*citri*	Subandiyah et al 2000
EU563934	Proteobacteria	Betaproteobacteria	Burkholderia vietnamiensis	Endo (plant)	Malpighiales	Salicaceae	*Populus*	*trichocarpa*	Xin et al 2009
DQ449047	Bacteriodetes	Sphingobacteria	Cardinium sp.	Endo	Actinedida	Tetranychinae	*Tetranychus*	*cinnabarinus*	Liu et al 2006
CGU41346	Bacteriodetes	Flavobacteria	Capnocytophaga gingivalis	Env					Vandamme et al 1996

AB078047	Bacteriodetes	Flavobacteria	Flavobacterium columnare	Env	Nakagawa et al 2002
AF289153	Bacteriodetes	Sphingobacteria		Env	Zwart et al 2002
EU887816	Firmicutes	Clostridia	Tissierella praeacuta	Env	Nayak and Kale 2008
DQ129384	Undetermined			Env	Andersen et al. 2007
HQ674328	Undet			Env	Wright and Hallam 2010
NR024570	Proteobacteria	Gammaproteobacteria	Escherichia coli	Env	Cilia et al 1996
FJ528593	Firmicutes	Bacillis	Lysinicacillus sphaericus	Env	Ding et al 2008 unpub
AM237373	Proteobacteria	Gammaproteobacteria	Soldalis glossinidius	Env	Alenius et al 2009
NR043552	Proteobacteria	Betaproteobacteria	Burkholderia oklahomensis	Env	Glas et al 2011 unpub
DQ108390	Proteobacteria	Betaproteobacteria	Burkholderia oklahomensis	Env	Glas et al 2011 unpub
DQ108391	Proteobacteria	Betaproteobacteria	Burkholderia oklahomensis	Env	Glas et al 2011 unpub
DQ108389	Proteobacteria	Betaproteobacteria	Burkholderia oklahomensis	Env	Glas et al 2011 unpub
HM449983	Undet			Env	Gruwell et al 2010
DQ388535	Proteobacteria	Betaproteobacteria	Burkholderia thailandensis	Env (pathogen)	Glass et al 2006
AY741347	Proteobacteria	Betaproteobacteria	Burkholderia cepacia	env	Lilburn et al 2004 unpub
GU119230	Undet			Env	Sungawa et al 2010
JF958162	Proteobacteria	Betaproteobacteria	Burkholderia sp.	Env	Chun 2011 unpub
HQ327182	Undet			Env	Zhao and Ma 2010 unpub
JF833519	Proteobacteria	Betaproteobacteria		Env	Wang 2011 unpub
HM063869	Proteobacteria	Betaproteobacteria	Ralstonia sp.	Env	Bruce et al 2010
HQ230116	Undet			Env	Hardin et al 2010
FJ193804	Proteobacteria	Betaproteobacteria	Janthinobacterium	Env (space)	Varishampayan et al 2008
AY959043	Undet			Env (pathogen)	Hyman et al 2005
EU024141	Proteobacteria	Betaproteobacteria	Comamonas sp.	Env	Ait et al 2007
EU799537	Undet			Env	Shaw et al 2008
NR026527	Chlamydiae	Chlamydiae	Chlamydia	Env (pathogen)	Wilson et al 1995